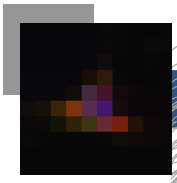

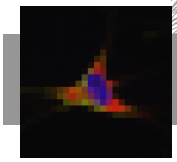

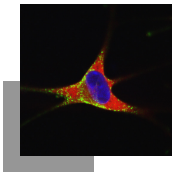

UNIVERSITEIT GENT

FACULTY OF **MEDICINE AND HEALTH SCIENCES**

THE NF1 GENE AND ITS GENE PRODUCT NEUROFIBROMIN

towards a functional role in human epidermal melanocytes

JOACHIM BOUCNEAU

2006

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The NF1 gene and its gene product neurofibromin: towards a functional role in human epidermal melanocytes

Thesis submitted as partial fulfillment of the requirements for the degree of Doctor in Medical Sciences

by

JOACHIM BOUCNEAU

2006

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Image(s) on the cover

Primary human epidermal melanocyte of a Neurofibromatosis type 1 (NF1) patient, immunostained for neurofibromin (red), the melanosomal transmembrane glycoprotein gp100 / NKI-beteb (green) and the nucleus (blue). The image was captured with a BioRad Radiance 2100 Blue Diode Confocal Laser Scanning Microscope.

‘It is the tension between creativity and skepticism
that has produced the stunning and unexpected findings of science’

Carl Sagan

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List of Abbreviations

aa	amino acid
AC	adenylate cyclase
ACTH	adrenocorticotropic hormone
bFGF	basic fibroblast growth factor
bHLH	basic helix-loop-helix
bp	base pair
CALM	café-au-lait macule(s)
cAMP	cyclic adenosine monophosphate
CBP	CREB binding protein
cDNA	complementary DNA
CHS	Chediak-Higashi syndrome
CRE	cAMP responsive element
CREB	cAMP responsive element binding protein
CSRD	cysteine / serine rich domain
CT	cholera toxin
CTD	carboxyterminal domain
DCT	dopachome tautomerase
DDD	dead de-epidermized dermis
DNA	deoxyribonucleic acid
EDN1	endothelin 1
EDN3	endothelin 3
EDNRB	endothelin receptor B
EMU	epidermal melanin unit
ERK	extracellular signal-regulated protein kinase
EVI2A	ecotropic viral insertion site 2 protein A
EVI2B	ecotropic viral insertion site 2 protein B
GAP	GTPase activating protein
GEF	guanine nucleotide exchange factor
GM-CSF	granulocyte / macrophage colony-stimulating factor
GRD	GAP related domain
GS	Griscelli syndrome
GSK3β	glycogen synthase kinase 3β
HGF	hepatocyte growth factor
HMG	high mobility group
HPS	Hermansky-Pudlak syndrome
IBMX	3-isobutyl-1-mehtylxanthine
kb	kilobase
kDa	kilodalton
LEF1	lymphoid enhancer-binding factor 1
LZ	leucine zipper
MAPK	mitogen activated protein kinase
MC1R	melanocortin 1 receptor
MITF	microphthalmia-associated transcription factor
MPNST	malignant peripheral nerve sheet tumor
mRNA	messenger RNA
NF1	neurofibromatosis type 1
NLS	nuclear localization signal
OCA	oculocutaneous albinism
OMGP	oligodendrocyte-myelin glycoprotein
PAX3	paired box 3
PCR	polymerase chain reaction
PI3K	phosphatidylinositol-3-kinase
PKA	cAMP-dependent protein kinase A
PKB	protein kinase B
qPCR	quantitative PCR
RNA	ribonucleic acid
RT-PCR	reverse transcriptase PCR
SCF	stem cell factor
SER	smooth endoplasmic reticulum
SOS	son-of-sevenless
SOX10	SRY-related HMG-box 10
TDE	tyrosinase distal element
TGN	trans-Golgi network
TPE	tyrosinase proximal element
TYR	tyrosinase
TYRP1	tyrosinase-related protein 1
TYRP2	tyrosinase-related protein 2
UV	ultra-violet
WS1-4	Waardenburg syndrome type 1-4
α-MSH	α-melanocyte stimulating hormone

Introduction

The melanocyte: general biology

1 Structural and functional aspects

1.1 The melanocyte: key player of the epidermal melanin unit

In vivo, the pigment producing cell, known as the melanocyte, resides in the basal layer of mammalian epidermis (see Figure 1) in a multicellular association with approximately 36 viable keratinocytes (Fitzpatrick and Breathnach, 1963). Within this so-called functional epidermal melanin unit (EMU) the melanocyte synthesizes, transports and transfers the pigment melanin to its surrounding keratinocytes (Quevedo et al., 1975). The various amounts and types of melanins (see p.14) in the recipient keratinocytes significantly determine the color of mammalian skin and hair.

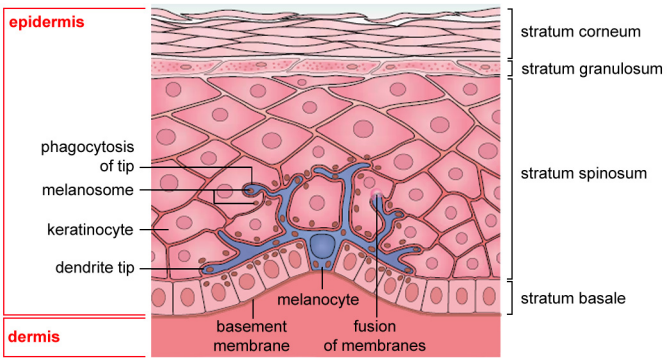

Figure 1. Representation of the epidermal melanin unit (EMU). A melanocyte resides in the stratum basale of the epidermis. In normal skin approximately every tenth cell, anchored on the basement membrane, is a melanocyte. Melanosomes are transferred from the melanocyte into surrounding keratinocytes of the EMU. Keratinocyte stem cells (stratum basale) divide and daughter cells undergo terminal differentiation while being pushed upwards in the different epidermal layers. The dermo-epidermal junction consists of an undulating basement membrane that adheres the dermis to the epidermis (adapted from 'Dermatology', chapter 65: Biology of melanocytes, by Bolognia & Orlow, 2003).

Pigmentation of the skin has important evolutionary and physiological implications with photoprotection of underlying tissues from ultraviolet (UV)-induced DNA damage being the most valuable function (Hill, 1992). It has been shown that eumelanins (see p.14) not only function as 'sunscreen' but that other properties, like anti-oxidant and free radical scavenging, are also important in cell and tissue protection (Prota, 1994; Kobayashi et al., 1995).

1.2 The melanosome: a unique pigment cell-specific organelle

The melanosome is a unique melanocytic organelle in which melanin is synthesized and deposited. Melanosomes undergo a well-defined, sequential, multistep maturation process. The melanosome is a hybrid organelle that initiates from the fusion of premelanosomes, originating from the smooth endoplasmic reticulum (SER), and tyrosinase-loaded vesicles, budding off from the trans-Golgi network (TGN). This dual vesicular origin is known as the bipartite theory of melanosome biogenesis (Jimbow et al., 1976; Kushimoto et al., 2001; Basrur et al., 2003). These so-called stage I (pre)melanosomes transform into elongated, fibrillar organelles which are tyrosinase-positive (stage II melanosomes). From here on melanin biosynthesis begins and pigment granules are uniformly deposited onto intralumenal fibrillic structures, giving the melanosome a darkened striated appearance (stage III melanosomes). In heavily pigmented melanocytes, melanin synthesis and deposition continues until no intralumenal structural details can be seen (stage IV melanosomes) (Seiji et al., 1963; Hearing, 2005). During and following this maturation process, melanosomes are transported from their site of origin (perinuclear cytosplasm), through elongated processes, called dendrites, towards the dendritic tips of the melanocyte. The process is necessary for the subsequent transfer to keratinocytes in the EMU. This intracellular organelle translocation process relies on several molecular motor proteins in close association with the microfilament (actin) and microtubule (tubulin) cytoskeleton (reviewed in Lambert et al., 1999; Westbroek et al., 2001). Long-range anterograde melanosome transport (perinuclear to peripheral) along the microtubules is mediated by the motor protein kinesin and is an ATP-hydrolysis driven process (Vancoillie et al., 2000b; Hara et al., 2000). Kinesin binds directly to kinectin which is a melanosomal membrane-associated protein and believed to be a receptor for kinesin (Vancoillie et al., 2000c). The retrograde movement of melanosomes (peripheral to perinuclear) is mediated by the motor protein dynein that can bind the protein complex dynactin which is associated on the melanosomal membrane (Vancoillie et al., 2000a). Melanocytic dendrites consist of a central microtubule core surrounded by a subcortical actin network (Lacour et al., 1992). In order to obtain a correct peripheral melanosome distribution, a ‘cooperative delivery and capture’ model was proposed (Wu et al., 1998). The motor protein-mediated (kinesin and dynein) melanosome transport back-and-forth the microtubule cytoskeleton in the dendrites is followed at the appropriate time by melanosome capture into the subcortical actin network by the F-actin associated motor protein myosin Va (Lambert et al., 1998). It was shown that only myosin Va isoforms containing exon F were able to perform this process in melanocytes, and that a tight tripartite protein complex between myosin Va (containing exon F), melanophilin and Rab27a (the latter being a GTP-binding protein and present on the melanosomal membrane) was necessary to mediate this translocation (Westbroek et al., 2003). Mutations in myosin Va and in Rab27a were shown to impair this melanosome transport (Bahadoran et al., 2003), causing pigmentary dilution as found in Griscelli syndrome type 1 (GS1; Pastural et al., 1997) and type 2 (GS2; Menashe et al., 2000) patients, respectively. After melanosomes are captured in the peripheral dendritic subcortical actin network, they must be transferred to the surrounding epidermal keratinocytes, where they form supranuclear melanin caps (Sulaimon and Kitchell, 2003). Much less is known about the molecular events regulating this process. Several hypotheses have been proposed and several approaches have been established to monitor the transfer process (Berens et al., 2005). The cytophagocytosis theory states that a melanocytic cell part, being the tip of a melanocyte dendrite or filopodium containing melanosomes, is pinched off and phagocytosed by a keratinocyte (Okazaki et al., 1976; Yamamoto and Bhawan, 1994). The discharge theory describes melanin release in the extracellular space by exocytosis (fusion of melanosomal membrane and melanocyte plasma membrane). Subsequent melanin is taken up by the recipient keratinocyte by phagocytosis (Virador et al., 2002). The fusion theory defines fusion of both keratinocyte and melanocyte plasma membranes forming a tunnel structure through which transport of melanosomes occurs (Scott et al., 2002). Finally, direct inoculation of melanosomes in keratinocytes has been proposed.

2 Melanocyte development and proliferation

In human embryos neural crest migration is complete by 7 weeks and melanoblasts, the neural-crest derived melanocyte precursors, start to appear in the dermis (Zimmerman and Becker, 1959). Shortly after, they migrate into the epidermis to their final location on the basal membrane of the basal epithelial layer of the epidermis (dermo-epidermal junction; Mayer, 1973). By 10 weeks epidermal melanocytes are observed to have premelanosomes (Fujita et al., 1970). The final number and distribution of melanocytes in the epidermis is quite constant (Holbrook et al., 1988). The developmental proces (migration and differentiation) of the melanocyte lineage is strictly controlled and most information is discernible based on available mutants (reviewed in Boissy and Nordlund, 1997; see also p.20). Migration of melanoblasts occurs along the dorsolateral pathway, between the somites and ectoderm, and is mainly regulated by (spatiotemporal) action of the KIT ligand / KIT receptor complex (reviewed in Yoshida et al., 2001; Kunisada et al., 2001; Wehrle-Haller, 2003).

Regulation of melanocyte cell growth is mainly controlled by external (keratinocyte-derived) growth factors and indicates that a balanced activation of cell surface events is critical for normal melanocyte proliferation and development. Peptide growth factors like basic fibroblast growth factor (bFGF), hepatocyte growth factor (HGF), stem cell factor (SCF; KIT ligand), endothelins (EDN) and melanotropins are known mitogens for human melanocytes. In vitro, normal human melanocytes require synergistic mitogens in addition to common growth factors in order to proliferate. Any disturbance of the normal function of these growth factors and their cognate receptors, by elimination due to deletions or mutations or by over-stimulation due to ectopic expression, has a direct impact on the number and distribution of melanocytes in vitro and in vivo (Halaban, 2000; see also p.21).

3 Melanocyte differentiation: cellular, biochemical and molecular aspects

3.1 The melanin biosynthesis pathway

Melanins represent a group of complex polymorphous, nitrogenous, multifunctional biopolymers whose function vary from camouflage to the quenching of oxidative free radicals after UV light exposure. The two major forms of melanin in skin and hair are the brown-black eumelanins and the yellow-red pheomelanins. Both melanins can be synthesized and cross-polymerized simultaneously and are known as mixed melanins. Similar to these melanins are the brown-black neuromelanins found in deep brain regions. They are by-products of dopamine oxidation within neurons and are believed to act as iron sinks (Zecca et al., 2001). A common property of all melanins is that several melanin units are linked by strong carbon-carbon (C-C) bonds, but that they differ from one another in structural and physical characteristics, as well as chemical composition (Prota, 1995; Ito, 2003).

The amino acid L-tyrosine is the precursor for the production of all melanins. Being a complex multistep, chemical and enzyme-mediated transformation process, the melanin biosynthesis pathway (see Figure 2) initiates with the hydroxylation of L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA). This step is obligatory both in vitro and in vivo. L-DOPA can also serve as a precursor to both melanins and catecholamines (dopamine, norepinephrine and epinephrine), acting along seperate pathways. In the eumelanogenic pathway L-DOPA is oxidized to dopaquinone, followed by rearrangements to leucodopachrome and then to dopachrome. A series of oxidoreduction reactions leads to the production of the intermediates 5,6-dihydroxyindole-2-carboxylic acid (DHICA) and 5,6-dihydroxyindole (DHI). These are then further oxidized to indole-5,6-quinone-2-carboxylic acid and indole-5,6-quinone, respectively, and undergo polymerization to form a poorly soluble, brown DHICA-melanin and an insoluble, black DHI-melanin, respectively (Prota, 1995; Ito, 2003).

The eumelanogenic and pheomelanogenic pathway diverge in the dopaquinone step (Prota, 1995). In the latter pathway dopaquinone gets conjugated with gluthatione and / or the amino acid L-cysteine to form gluthationyl-

and cysteinyldopa, respectively. These intermediates are converted into alanyl-hydroxy-benzothiazine units which are then polymerized into soluble, yellow-red pheomelanin.

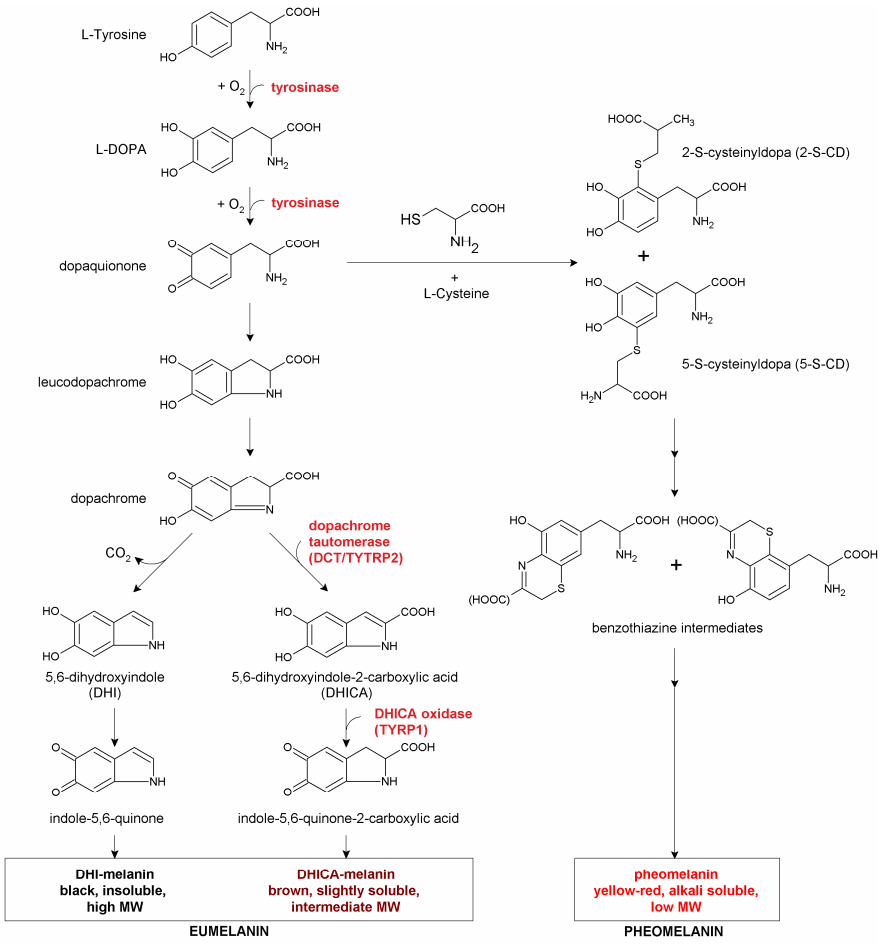

Figure 2. The melanin biosynthesis pathway. Tyrosinase is the key enzyme giving rise to L-3,4-dihydroxyphenylalanine (L-DOPA) and dopaquinone, through hydroxylation of the precursor L-tyrosine. Eumelanins are derived from dopachrome metabolites, 5,6-dihydroxyindole (DHI) and 5,6-dihydroxyindole-2-carboxylic acid (DHICA). The latter compound is sequentially synthesized through action of the enzymes dopachrome tautomerase (DCT) or tyrosinase-related protein 2 (TYRP2), and DHICA oxidase or tyrosinase-related protein 1 (TYRP1). Pheomelanins are derived from other metabolites in the extra presence of L-cysteine.

3.2 The pigmentary genes and their products: the tyrosinase gene family

Several genes (127 loci described untill now in mice) are involved directly or indirectly in the regulation of pigment production. However, it is beyond the scope of this chapter to describe them all. A complete list of the coat color genes in mice and their human homologs can be accessed at http://ifpcs.med.umn.edu/micemut.htm.

Here we will focus on the genes and their translation products involved in the enzymatic regulation of eu- and pheomelanogenesis (see p.14). The tyrosinase gene family includes the genes coding for tyrosinase (TYR), tyrosinase related protein 1 (TYRP1; DHICA oxidase) and tyrosinase related protein 2 (TYRP2; dopachrome tautomerase or DCT). These gene products show a high degree of homology and share common structural domains: (i) an amino-terminal signal sequence important in correct protein processing and intracellular trafficking (Oetting et al., 1994), (ii) two cysteine-rich domains of which the first contains an epidermal growth factor (EGF)-like protein binding region (Jackson et al., 1992; Sturm et al., 1995) which is potentially important in forming a functional multiprotein melanogenic enzyme complex within the melanosome (Orlow et al., 1994), (iii) two putative copper-binding sites, (iv) a highly hydrophobic transmembrane domain important for anchorage in the melanosomal membrane and (v) a short cytoplasmic tail. The latter two domains are important for the correct

intracellular targeting of the enzyme and do not play a role in the catalytic function of the enzymes. The initial steps in melanin formation, i.e. hydroxylation of L-tyrosine to DOPA (which is the rate-limiting step) and oxidation of DOPA to dopaquinone, are mediated by tyrosinase. TYRP1 oxidizes DHICA to quinones and is found distal of tyrosinase and TYRP2 in the melanin biosynthetic pathway. TYRP2 mediates the conversion of dopachrome to DHICA. More on the transcriptional regulation of these melanogenic genes is shown on p.18. More on genetic disorders caused by mutations in genes of the tyrosinase gene family is described on p.20.

3.3 The melanocyte signal transduction pathways

The molecular processes involved in the regulation of melanogenesis are centered around two major signal transduction pathways: the cyclic AMP (cAMP) pathway and the receptor tyrosine kinase c-KIT / mitogen activated protein kinase (MAPK) cascade pathway (see Figure 3). These pathways play a pivotal role in regulating expression and / or activity of melanogenic enzymes. Regulation of the expression of melanogenic enzymes mainly implies stimulation through a tightly controlled transcriptional mechanism (see p.18).

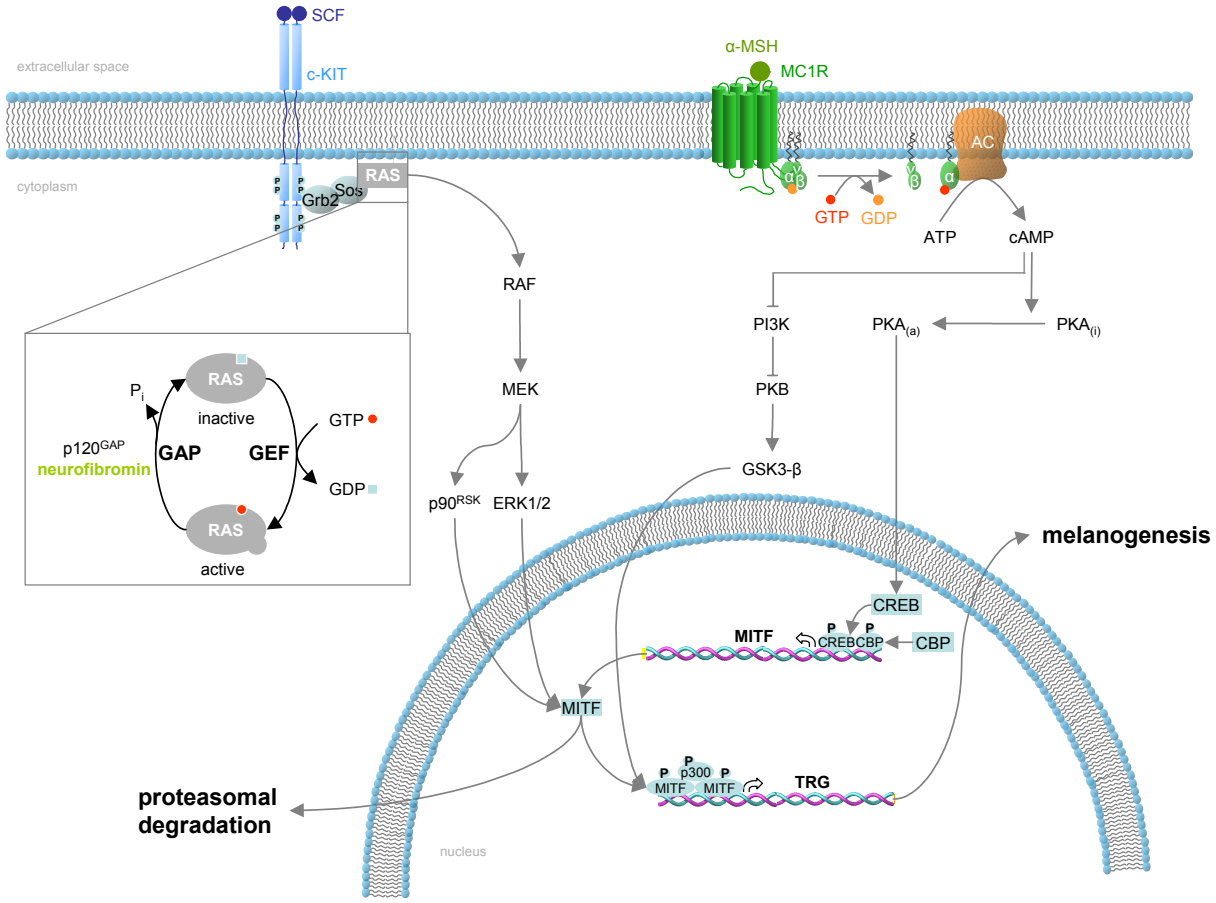

Figure 3. Signal transduction pathways involved in melanogenesis. Two major signal transduction pathways are involved in regulation of melanogenesis: the cAMP pathway and the c-KIT / $p21^{Ras}$ / Raf / Mek / Erk pathway. Both pathways can be triggered by extracellular signals activating their respective receptors. TRG, tyrosinase-related genes; (i), inactive; (a), active.

The cyclic AMP pathway

In melanocytes extracellular (melanogenic) stimuli like the pro-opiomelanocortin peptides α-melanocyte stimulating hormone (α-MSH) or adrenocorticotropic hormone (ACTH), derived from surrounding epidermal keratinocytes, play a pivotal role in melanocyte function. These ligands can bind to the melanocortin-1 receptor (MC1R), a seven transmembrane domain receptor coupled to a heterotrimeric $G_{\alpha s}$-protein (Barsh, 1996). Upon

ligand-receptor complex formation, $G_{\alpha s}$-protein activates its downstream effector adenylate cyclase (AC), resulting in a significant increase of the intracellular second messenger cyclic AMP (cAMP). The upregulation of melanogenesis can be mimicked by pharmacological cAMP elevating agents such as forskolin, cholera toxin (CT) or 3-isobutyl-1-mehtylxanthine (IBMX) (Hearing and Tsukamoto, 1991; Englaro et al., 1995). cAMP induces a complex set of intracellular interconnected processes of which the activation of cAMP-dependent protein kinase A (PKA) is the leading sub-pathway towards melanogenesis (Lalli and Sassone-Corsi, 1994; Bertolotto et al., 1996). Phosphorylated (activated) PKA is then translocated to the nucleus where it phosphorylates the cAMP responsive element binding protein (CREB) family of transcription factors and CREB binding proteins (CBP). Genes containing a consensus CREB responsive element (CRE) sequence in their promotor are direct targets of these transcription factors (Karin, 1994). One of those genes is the microphthalmia-induced transcription factor (MITF) of which transcription is upregulated in a PKA-dependent fashion. MITF is able to bind to the E-box and M-box sequences in the promotor of the melanogenic genes tyrosinase, TYRP1 and TYRP2 (Bertolotto et al., 1998). Their increased expression leads to upregulation of melanin biosynthesis. Beyond this above scheme, it has been shown that cAMP can regulate melanogenesis through a PKA-independent mechanism. cAMP can inhibit phosphatidylinositol-3-kinase (PI3K), resulting in inhibition of protein kinase B (PKB / AKT) phosphorylation and activity. Upon PKB inhibition, glycogen synthase kinase 3β (GSK3β) gets dephosphorylated and activated after which it is able to phosphorylate MITF on serine 208 (Ser-208), increasing the ability of MITF to bind and transactive the tyrosinase promotor (Busca and Bertolotto, 1996; Takeda et al., 2000; Khaled et al., 2002). Elevating cAMP levels leads to activation of a complex network of signaling pathways that diverge in cAMP and converge to MITF to control melanin synthesis and melanocyte differentiation.

The receptor tyrosine kinase c-KIT / MAP kinase cascade pathway

The receptor c-KIT plays a pivotal role in melanocyte development and differentiation, illustrated by the fact that patients with a heterozygous c-KIT mutation develop a pigmentary disorder with amelanotic skin patches, called piebaldism (Spritz et al., 1993). In response to its ligand SCF, c-KIT signaling activates the MAPK cascade and triggers two phosphorylation events on MITF. Activated extracellular signal-regulated protein kinase 1 and 2 (ERK1/2) are translocated to the nucleus where they directly phosphorylate MITF at serine 73 (Ser-73), while serine 409 (Ser-409) serves as substrate for $p90^{RSK1}$ (Wu et al., 2000). This c-KIT-induced dual phosphorylation upregulates the transactivational potential of MITF yet simultaneously couples it to proteasome-mediated degradation by targeting the protein to ubiquitin-dependent proteolysis. This coupled activation / degradation signal seems paradoxical in the regulation of pigmentation. It has been shown that MITF transactivates the tyrosinase, TYRP1 and TYRP2 melanogenic gene promotors (Hemesath et al., 1994; Yasumoto et al., 1994; Bertolotto et al., 1996, Bertolotto et al., 1998) and that c-KIT stimulation recruits the transcriptional co-activator p300/CBP to MITF (Price et al., 1998). Transactivation activity is markedly enhanced by MAPK-mediated activation of MITF (Hemesath et al., 1998). However, dominant active and dominant negative $p21^{Ras}$ did not increase or reduce pigmentation, respectively (Englaro et al., 1998), despite evidence that c-KIT upregulated MITF through ERK1/2 (Hemesath et al., 1998). Probably the same signaling pathway may target MITF for activation as well as proteolytic degradation. Homeostatic downregulation is a hallmark of receptor tyrosine kinase signaling pathways and occurs at multiple levels including cytokine / receptor internalization, dephosphorylation and proteolysis.

Link between two major melanogenesis-regulating signal transduction pathways

In many cell types, cAMP has been shown to inhibit the MAPK cascade. However, it has been demonstrated in B16 mouse melanoma cells and human melanocytes that elevation of cAMP levels activates ERK1/2 (Englaro et

al., 1995). This cAMP-dependent activation of ERK1/2 is mediated by the activation of $p21^{Ras}$ and B-RAF kinase, and seems to be cell-specific and independent of PKA. However, the classical $p21^{Ras}$ guanine nucleotide exchange factors (GEF) Son-of-sevenless (SOS) and Epac are not involved in cAMP-dependent $p21^{Ras}$ activation, which suggests the existence of a novel melanocyte-specific cAMP-dependent $p21^{Ras}$ GEF (Busca et al., 2000).

3.4 Transcriptional regulation of melanogenesis

It is clear that multiple factors, including ubiquitous and cell-type specific factors, have to cooperate to direct transcription of the tyrosinase gene family. This provides a good system to study mechanisms for cell-type specific gene transcription. Neural-crest derived melanocyte-specific gene expression is tightly controlled by the interplay between specific signal transduction pathways (see p.16) and transcription factors. In the center of transcriptional regulation of melanocyte differentiation is MITF, a basic helix-loop-helix leucine zipper (bHLH-LZ) transcription factor residing in multiple signaling pathways important to the melanocyte-lineage. Heterozygous MITF mutations exhibit pigmentary defects due to reduced melanocytes in the skin and hair, a condition known as Waardenburg syndrome type 2A (see p.20). The control of expression of melanocyte-specific MITF (MITF-M) is regulated by an array of transcription factors that can bind to its promotor (Fuse et al., 1996). CREB and related factors confer on the MITF-M promotor responsiveness to cAMP and MC1R signaling (Bertolotto et al., 1998; Price et al., 1998). It has been shown that MITF is required but not sufficient to induce melanogenic gene expression (Gaggioli et al., 2003). The high-mobility group (HMG) box protein SRY-related HMG-box 10 (SOX10) has been shown to bind to the proximal promotor (Lee et al., 2000) and an upstream enhancer of MITF (Watanabe et al., 2002) and can cooperate, although not interact, with MITF in activating the TYRP2 promotor (Ludwig et al., 2004; Jiao et al., 2004). The paired homeobox domain factor 3 (PAX3) has been shown to regulate MITF expression via consensus elements within the MITF promotor (Watanabe et al., 1998; Potterf et al., 2000). The lymphoid enhancer factor 1 (LEF1) transcription factor interacts with ß-catenin and enables MITF expression to be regulated by Wnt signaling. It cooperates with MITF, through interaction, in the activation of the TYRP2 promotor (Yasumoto et al., 2002). The human TYR gene contains an enhancer element called the tyrosinase distal element (TDE) containing a CATGTG motif at its center and is located 1.8 kb upstream of the transcription initiation site. More proximal (-112 to –93), an additional copy of this CATGTG motif is found as tyrosinase proximal element (TPE) (Yasumoto et al., 1994). Together with the TDE and TPE, there are two additional proximal positive elements, called the M-box (included in the TPE) and the initiator E-box, showing to be the binding site for MITF in transactivation of the human TYR promotor (Bentley et al., 1994; Yasumoto et al., 1997). A region similar to TDE was found more proximal in the TYRP1 promotor (Yasumoto et al., 1994). The TYRP2 promotor contains two putative cis-acting elements similar to the M-box and a proximal melanocyte-specific promotor (Shibahara et al., 1991).

The melanocyte dysregulated: pigmentary disorders

Mammalian melanogenesis, starting from migration of precursor cells to catabolism of melanin in the skin, is summarized in several steps in Table 1.

Table 1. Mammalian melanogenesis (adapted from Sulaimon and Kitchell, 2003).

Step I	Melanoblasts migrate from the neural crest into the epidermis
Step II	Melanoblasts differentiate into melanocytes and undergo clonal expansion in the skin
Step III	Pre(melanosome) matrix formation
Step IV	Melanogenic genes (tyrosinase, tyrosinase related proteins and melanosomal matrix components) induced
Step IV	Tyrosinase and related melanogenic proteins synthesized
Step V	Posttranslational processing and glycosylation of tyrosinase
Step VI	Fusion of vesicles to form melanosomes and initiation of melanogenesis
Step VII	Control of tyrosinase activity
Step VIII	Control of the activity of tyrosinase related protein
Step IX	Modification of melanin
Step X	Melanosome transfer to keratinocytes
Step XI	Melanosome degradation
Step XII	Melanin removal with loss of cornified cell

The major determinant of normal skin color is the quantity and quality of melanin present in epidermal keratinocytes and is the resultant of increased melanocyte activity (synthesis, transport, transfer). The density of epidermal melanocytes has no influence on constitutive pigmentation. In order to understand the underlying etiology and pathophysiology of cutaneous disorders of hypo- and hyperpigmentation, knowledge of structural, functional and genetic determinants in development, survival, migration, proliferation and differentiation of melanocytes is of crucial importance. Several pigmentary disorders are linked to specifc gene defects, while others are the result of imbalances in para- and / or autocrine cytokine networks, or a combination of both. However, it is beyond the scope of this chapter to address all disorders of hypo- and / or hyperpigmentation. Relevant examples will be given to illustrate the underlying principles and will be largely based on the genetic and structural components discussed in the above chapters.

1 Genetic disorders of pigmentation

1.1 Heterogenous ("spotty") pigmentary defects: impaired melanocyte development

Pigmentary anomalies that are characterized by a heterogenous distribution of melanin pigments (congenital pigment spots) are mostly associated with abnormal distribution of melanocytes during embryological development. The receptor tyrosine kinase c-KIT has been shown to be defective in cases of human piebaldism, suggesting a role for the SCF / c-KIT signaling pathway (see p.16) to be involved in melanocyte development (Giebel and Spritz, 1991). This ligand-receptor complex seems to be crucial for melanocyte survival throughout development until late stages of differentiation in the skin (Wehrle-Haller, 2003). Another ligand-receptor system, between the endothelin receptor B (EDNRB) and its ligand endothelin-3 (EDN3), is important for early expansion and migration of melanoblasts while delaying differentiation (Lahav, 2005). Mutations in EDN3 lead to Waardenburg syndrome type IV (WS4) (Edery et al., 1996; Hofstra et al., 1996), while defective EDNRB is the cause of Hirschsprung disease (Attie et al., 1995). Two transcription factors have been implicated in the development of melanocytes based on either similarity to mouse mutants: MITF, defective in Waardenburg syndrome type II (WS2) (Hughes et al., 1994) and PAX3, defective in Waardenburg syndrome type I and III (WS1/3) (Tassabehji et al., 1993; Baldwin et al., 1995). In addition to its evident role in melanocyte development, MITF also appears to play a critical role in regulating melanocyte function by controlling transcriptional activation of the melanogenic genes (see p.15 and p.18). PAX3 seems to be critical for activating melanoblasts and other cellular elements to proliferate or to initiate migration from the neural crest (Stuart and Gruss, 1995).

1.2 Homogenous pigmentary defects: impaired melanocyte function

Deficient melanin biosynthesis and processing can lead to homogenous increase or reduction of pigmentation and is characteristic for genetic disorders of melanocyte function. It is beyond the scope of this chapter to discuss them all. Here we will shortly focus on the most common inherited disorders of generalized hypopigmentation known to human, of which albinism is the most frequent and well-known (see Table 2).

Table 2. Pigmentary disorders characterized by diffuse pigment dilution in which the gene defect is known.

Disorder	Gene	Protein product	References
Oculocutaneous albinism (OCA)			
type 1A	TYR	tyrosinase	Spritz et al., 1989
type 1B	TYR	tyrosinase	Giebel et al., 1991
type 2	P	P protein	Lee et al., 1994
type 3	TYRP1	TYRP1	Boissy et al., 1996
Hermansky-Pudlak syndrome			
type 1	HPS1	novel protein	Fukai et al., 1995
type 2	AP3B1	β3A subunit of AP3	Dell'Angelica et al., 1999
type 3	HPS3	novel protein	Anikster et al., 2001
type 4	HPS4	novel protein	Suzuki et al., 2002
type 5	HPS5	novel protein	Zhang et al., 2003
type 6	HPS6	novel protein	Zhang et al., 2003
type 7	DTNBP1	dystrobrevin binding protein 1	Li et al., 2003
Chediak-Higashi syndrome	CHS1 (LYST)	novel protein	Fukai et al., 1996

Table 2. (continued)

Disorder	Gene	Protein product	References
Griscelli syndrome			
type 1	MYOVA	myosin Va	Pastural et al., 1997
type 2	RAB27A	RAB27a	Menashe et al., 2000
type 3	MLPH	melanophilin	Menashe et al., 2003

2 Micro-environmental induced pigmentary defects: cytokine network imbalances

Paracrine melanogenic cytokine networks tightly regulate melanocyte function and the interplay between melanocytes and other types of skin cells (keratinocytes and fibroblasts) (Yada et al., 1991; Imokawa et al., 1998). In vivo, normal melanocyte homeostasis is the result of a fine expression, secretion and interaction balance of ligands and their cognate receptors, and subsequent signaling to the nucleus. A distortion in this fine balance, due to uncontrolled up- or downregulation of components of the cytokine network, is responsible for the constitutive activation or inactivation of melanocytes, which leads to hyperpigmentary or hypopigmentary defects, respectively. Many melanogenic paracrine networks exist of which imbalances are associated with distorted melanocyte proliferation and epidermal hyperplasia (see Table 3).

Table 3. Pigmentary disorders associated with melanogenic cytokine networks (adapted from Imokawa, 2004).

hypo- (□) / hyper- (■) pigmentary disorder	melanocyte proliferation	epidermal hyperplasia	intrinsic cytokine or chemokine or receptor	produced by	reference
UVB-melanosis (■)	↑	↑	EDN1 / EDNRB / mSCF / c-KIT	keratinocyte	Imokawa et al., 1995; Hachiya et al., 2001
UVA-melanosis (■)	↑	=	GM-CSF	keratinocyte	Imokawa et al., 1996
Riehl's melanosis (■)	↑	↗	GRO-α	keratinocyte	Imokawa et al., 1992
Lentigo senilis (■)	↑	↗	EDN1 / EDNRB / mSCF	keratinocyte	Kadono et al., 2001; Hattori et al., 2004
Dermatofibroma (■)	↑	↗	sSCF / HGF	fibroblast	Shishido et al., 2001
Café-au-lait macule (■)	↑	=	sSCF / HGF	fibroblast	Okazaki et al., 2003
Vitiligo vulgaris (□)	↓	=	c-KIT	melanocyte	Kitamura et al., 2004

EDN1, endothelin 1; EDNRB, endothelin receptor B; mSCF, membrane-bound stem cell factor; GM-CSF, granulocyte/macrophage colony-stimulating factor; GRO-α, growth related oncogene-α; sSCF, soluble stem cell factor; HGF, hepatocyte growth factor; ↑, increased; ↓, decreased; ↗, slightly increased; =, unchanged.

3 Microarray technology in the study of pigmentary defects: usefulness, critical considerations and evolution

Historically pigment cell research has been done one gene at a time. Current advances in molecular biology have made it possible to use genome-scale gene expression profile analysis to investigate thousands of genes simultaneously. The use of this high-throughput technology enables the discovery of (groups of) genes that are associated with a phenotypic difference. The best-known microarrays for gene expression profiling (=mRNA level) are the (c)DNA arrays. To accurately assess gene expression differences between normal and pathological

states, one should use tissue specimens derived from lesional tissue and compare them with the appropriate controls (Chen et al., 2003; Seykora et al., 2003). The use of cell lines or primary culture may be of limited benefit in elucidating cutaneous pathophysiology because these cells have been altered by removal from the native microenvironment (Seykora et al., 2003). However, all depends on the scientific question one wants to address (see **chapter 2**; Boucneau et al., 2005). As DNA microarrays are empirical screening tools, starting with a specific hypothesis is not always necessary. An investigator may perform a pilot experiment without a preconceived hypothesis. Following analysis of the initial results, a more discrete hypothesis can be formulated and hypothesis-driven experiments can be conducted (Dooley et al., 2003). The major critical determinants of a microarray experiment are its design, correct analysis and caution during interpretation (Loftus and Pavan, 2000). There are several sources of variation in microarray experiments. Biological variation is intrinsic to all organisms and might be influenced by genetic and / or environmental factors. Technical variation is introduced during preparation of the samples and during the subsequent labeling and hybridization steps. Finally, measurement errors are associated with reading the (fluorescent) signals. These sources of variation can be corrected for by using valid statistical tests and hence good designs should incorporate replication of experiments both at the biological and technical levels.

Microarrays are in their initial development for clinical application in a variety of tumor models. Melanoma is an ideal system to study the genetic changes associated with the stepwise progression of malignancy. It may be possible to efficiently screen the entire human genome to identify the particular aberrations in gene expression responsible for tumorigenesis in melanoma. Off course these applications can also be valuable tools in the search for unique pigmentary defect-associated markers that may be specifically expressed in cutaneous lesions (Seykora et al., 2003). The advent of protein-based microarrays allows the global observation of biochemical activities on an unprecedented scale, where thousands of proteins can be simultaneously screened for protein-protein, protein-nucleic acid, and small molecule interactions. This technology holds great potential for basic molecular biology research, disease marker identification, toxicological response profiling and pharmaceutical target screening (Bertone and Snyder, 2005). Together with gene / protein expression microarrays, which have highlighted many potential targets in cancer, tissue microarrays have emerged as a powerful tool to validate these targets by measuring tumor-specific protein expression and linking it to clinical outcome (Giltnane and Rimm, 2004). All these high-throughput technologies are becoming and will become invaluable tools in the molecular characterization and classification of all kinds of hypo- and hyperpigmentary disorders, not only in the lab but also in the clinic.

Neurofibromatosis type 1

1 Clinical features and diagnostic criteria

Neurofibromatosis type 1 (NF1), or von Recklinghausen neurofibromatosis, is a neurocutaneous monogenic disorder primarily affecting cells and tissues derived from the neural crest but many other organ systems as well. It is one of the most common autosomal dominant tumor predisposition syndromes known to humans with a worldwide estimated birth incidence of approximately 1 in 3500 individuals (Poyhonen et al., 1997). The pleiotropic NF1 disease manifestations (see Figure 4) result in a mild to severe clinical picture, even within families or patients with the same NF1 gene mutation (Friedman, 1999; Gutmann 2001). The clinical diagnostic criteria for NF1 are summarized in Table 4.

Table 4. Diagnostic criteria for NF1 (Stumpf et al., 1988; Gutmann et al., 1997).

The NF1 patient should meet 2 or more of the following criteria
Six or more café-au-lait macules (prepubertal: diameter ≥ 5 mm; postpubertal: diameter ≥ 15 mm)
Two or more neurofibromas (nodular and plexiform)
Multiple inguinal and / or axillary lentigines
Optic pathway glioma
Two or more Lisch iris hamartomas
Distinct osseous lesions (sphenoid dysplasia; thinning long bone cortex with / without pseudoarthrosis)
First-degree relative (parent, sibling or offspring) who meets the above criteria

Major disease manifestations

Café-au-lait macules are hyperpigmented patches of the skin that usually occur shortly after birth with a prevalence of around 95% in children younger than 1 year old. These hyperpigmentary spots are very striking features for NF1 but are not pathognomonic for the disease [see p.32; article I: Pigment-cell related manifestations of NF1: an overview].

Neurofibromas are benign multicellular tumors with a deranged tissue architecture of the peripheral nerves. These hamartomatous tumors are composed of several cells (Schwann cells, fibroblast, perineurial cells, endothelial cells and mast cells) forming the nerve sheath and excessive deposition of collagenous extracellular matrix

(Peltonen et al., 1984; Cichowsky and Jacks, 2001). The cutaneous (dermal) neurofibromas are soft, fleshy nodules and involve dermal and epidermal skin. They do not usually develop until preadolescence, with increasing tumor load during adolescent and young adult years (Huson, 1988; Riccardi, 1992a; North, 1993). Less than 15 percent of children younger than 10 years have cutaneous neurofibromas, compared to 44% in the 10-19 year age group, 85% of patients between 20-29 years of age, and 94% of adults over the age of 30 (North, 1993). Early appearance of neurofibromas may give some indication of the future severity of cutaneous manifestations for the individual patient; the earlier the onset, the more likely there will be a larger number and more extensive distribution of cutaneous neurofibromas (Riccardi, 1992b). In females there is often a clear history of an increase in number of neurofibromas during puberty and pregnancy (Dugoff and Sujansky, 1996), with an increase during puberty noted in the males. While cutaneous (dermal) neurofibromas may become a major cosmetic problem for individuals with NF1, they are not premalignant lesions and do not transform into malignant tumors (Gutmann et al., 1997). The subcutaneous (nodular) neurofibromas are more firm and involve peripheral nerves in deeper parts of the body. (Diffuse) plexiform neurofibromas occur in approximately 25% of patients with NF1 (Huson et al., 1988) and are of significant concern because of the potential for cosmetic disfigurement and malignant transformation (Friedman et al., 1999). Large lesions of the head and neck can cause major disfigurement and are present in 1-5% of patients. Diffuse plexiform neurofibromas often extend deeply to involve all levels of skin, muscle, bone and even viscera. They may cause compression, distortion or overgrowth of adjacent structures. Plexiform neurofibromas have a variable natural history in that some lesions may be quiescent for long periods. However growth may be aggressive and progressive, especially during infancy and the preadolescent / adolescent period. In general they are benign tumours. The value of surgical intervention for plexiform neurofibromas has been considered limited because the tumours do not follow tissue planes, are difficult to resect, are very vascular, and tend to regrow when incompletely removed. Plexiform neurofibromas, on the other hand, are usually congenital in origin and have the potential to undergo malignant transformation into malignant peripheral nerve sheath tumors (MPNST). These highly malignant neurofibrosarcomas are exceedingly rare in the general population and are a major cause of death in NF1 patients. Estimates of the frequency of peripheral nerve malignancy range between 1 and 4% (Huson et al., 1988; Riccardi, 1992b; North, 1993).

Axillary and inguinal freckling is the most useful criteria, after cafe-au-lait spots, for making the diagnosis of NF1 in young children since freckling tends to appear during the first five years of life, while neurofibromas and Lisch nodules may not appear until adolescence. The reported frequency of axillary freckling varies between 64% and 84% (Huson et al., 1988; Riccardi, 1992b). Inguinal freckling occurs in 52-56% of NF1 individuals (Huson et al., 1988; North, 1993). More generalized freckling may also occur on the trunk and neck, and in the submammary region in women [see p.32; article I: Pigment-cell related manifestations of NF1: an overview].

Lisch nodules are dome-shaped elevations of the iris surface and are pathognomonic of NF1. They do not cause symptoms but are extremely useful as a diagnostic tool, particularly in adults. The incidence of Lisch nodules in NF1 increases markedly with age; by the age of five years, around 50% of NF1 patients have Lisch nodules, whereas by over 30 years of age, 96-100% of patients have the lesions on slit lamp examination (Lubs et al., 1991; Ragge, 1993) [see p.32; article I: Pigment-cell related manifestations of NF1: an overview].

Minor disease manifestations and complications of NF1

Although not specific enough to warrant inclusion as diagnostic criteria, short stature and macrocephaly are common features of NF1. Approximately one-third of patients with NF1 have height at or below the third centile; short stature is not usually associated with growth hormone deficiency, abnormalities of the pituitary gland or structural skeletal defects. Almost 50% of individuals with NF1 have head circumference at or above the 97th

centile. Riccardi found a statistically significant difference between the distribution of height and head circumference centiles in patients with NF1 compared to the general population (Riccardi, 1992b). At present the mechanisms to account for these features of NF1 are unknown.

Optic pathway gliomas, also called optic nerve gliomas, represent the most common central nervous system (CNS) tumor in NF1 and are a specific feature of the disease (Listernick et al., 1997). MRI detects optic pathway gliomas in approximately 15% of NF1 patients. However, only 5% of these usually very benign tumors cause clinical symptoms (Lewis et al., 1984). Optic gliomas can involve any part of the visual pathway; the intraorbital portion of the optic nerves, the chiasm, the intracerebral visual pathways, or any combination thereof. Histologically, optic gliomas are low grade astrocytomas, not hamartomas (Alvord and Lofton, 1988) and it is not known why some tumors progress rapidly to cause symptoms while others remain quiescent for many years. Common symptoms of optic pathway tumours are decreased visual acuity, visual field defects and proptosis; other symptoms include optic atrophy, headache, nausea, anorexia and hypothalamic dysfunction (Habiby et al., 1995).

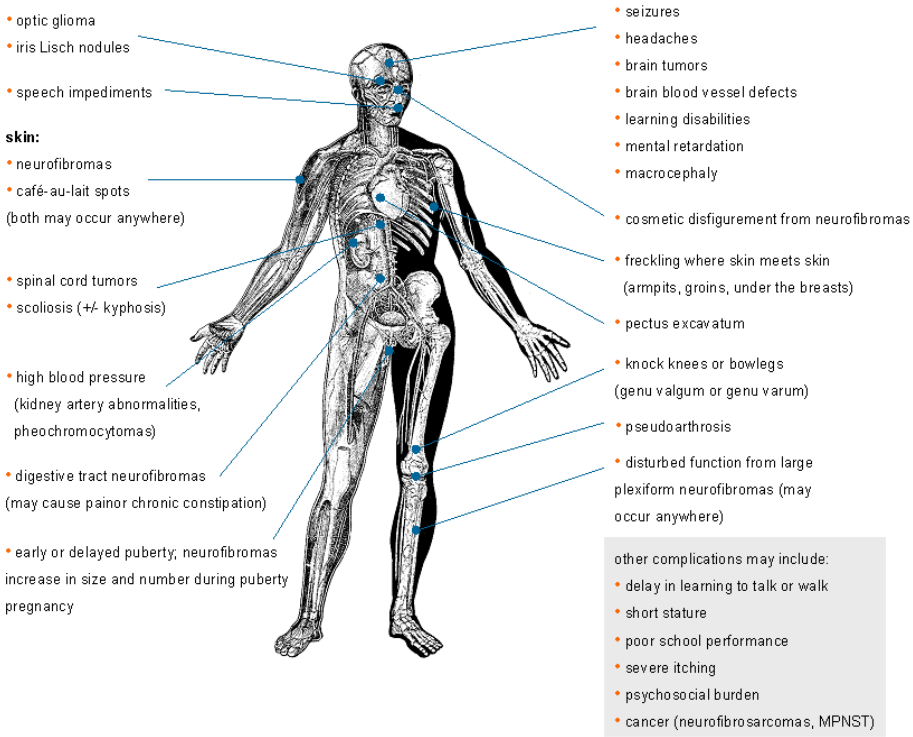

Figure 4. Overview of the clinical / phenotypic features of NF1 [adapted from Powell (1988)].

Distinctive osseous lesions, e.g. scoliosis, occurs in 12-20% of patients with NF1 (Huson et al., 1988; North, 1993). The majority of cases of scoliosis in NF1 resemble so-called 'idiopathic' scoliosis seen in the general population, with a long C-shaped curve involving a significant portion of the spine (up to 10 segments). A second, more severe type of scoliosis is seen specifically in NF1, and involves a smaller portion of the spine (less than five vertebrae) causing a very sharp, angular curve. This latter form may be associated with a localised area of vertebral dysplasia. Vertebral dysplasia may occur in isolation or secondary to a paravertebral neurofibroma; these 'benign' tumours appear to cause bony destruction, the mechanism of which is unknown (North, 1997). Children with NF1 tend to develop scoliosis at a younger age than other children, usually before the age of ten years.

The other complications of NF1 are individually rare, occurring in less than 5% of patients. These include epilepsy, intracranial tumours, hydrocephalus, pseudoarthrosis, sphenoid wing dysplasia, renal artery stenosis, and phaeochromocytoma.

2 NF1 gene structure

The NF1 gene was isolated by positional cloning and sequenced in the beginning of the 1990s (Cawthon et al., 1990b; Viskochil et al., 1990; Wallace et al., 1990). Initial linkage analyses mapped the gene to the proximal long arm of chromosome 17 and finally narrowed the location further down to about 3 cM of 17q11.2 (Barker et al., 1987; Goldgar et al., 1989). The gene has long been suspected to encompass approximately 350 kb of genomic DNA (Li et al., 1995). However, the latest entry (build 36.1: november 1, 2005) at the NCBI Map Viewer (http://www.ncbi.nlm.nih.gov/mapview) shows that the NF1 gene sequence only spans 280 kb. This is mainly due to the fact that at full sequencing of the first intron, it was shown that this intron is actually smaller (~60 kb) than previously estimated. The gene encodes a mRNA of 11-13 kb containing 59 exons (including alternative splice forms; see Figure 5) and is ubiquitously expressed in human tissues. An open reading frame of 8454 bp (Bernards et al., 1992; Marchuk et al., 1991) predicts a protein of 2818 aa with a calculated molecular weight of 327 kDa. Intron 27b contains three functional genes that are transcribed in opposite direction to NF1: OMGP (encoding oligodendrocyte-myelin glycoprotein), EVI2B (encoding ecotropic viral insertion site 2 protein B) and EVI2A (encoding ecotropic viral insertion site 2 protein A (Cawthon et al., 1990a; Cawthon et al., 1991; Viskochil et al., 1991). The AK3 pseudogene lies within intron 37 and is transcribed in the same orientation (Xu et al., 1992). The NF1 gene promotor is located in a CpG-rich region and does not

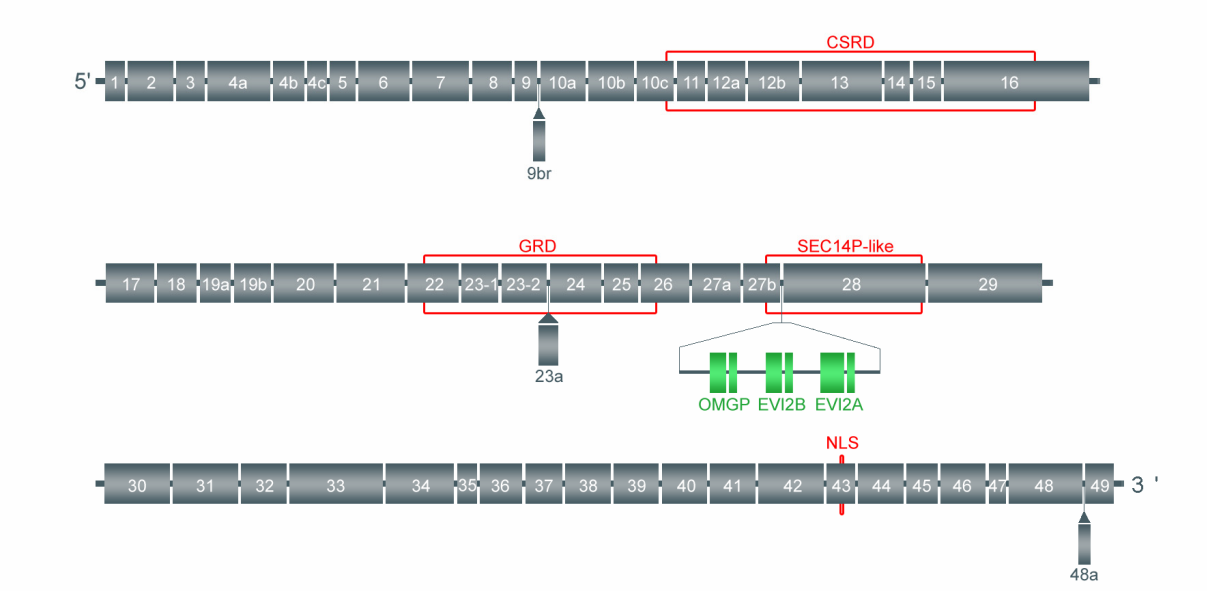

Figure 5. The NF1 gene structure. Schematic depiction of the NF1 gene (5'>3') showing representative exons (drawn in scale) with the known alternatively spliced exons (9br, 23a and 48a).The three coding genes (OMGP, EVI2B and EVI2A) inserted in opposite direction in the intron between exon 27b and exon 28 of the NF1 gene are shown in green. Three major domains / motifs (CSRD, GRD and NLS) are shown in red.

contain any strong transcriptional start site delineators (TATA / CAAT box). The transcriptional start site precedes the translational initiation site and locates 448 bp upstream of the latter. The 3' end of the NF1 gene is approximately 3.5 kb of genomic DNA downstream of exon 49. Several NF1 pseudogenes are scattered across the human genome and are thought to arise from duplication and transposition of the NF1 locus (Luijten et al.,

2000; Luijten et al., 2001). These NF1 related loci (e.g. on chromosome 2, 14 and 22) have been suggested as being disease-causing mutation carriers from which mutations can be transferred to the functional NF1 gene on chromosome 17 by interchromosomal gene conversion. The high mutation rate of the NF1 gene is one of the highest known to humans. Nearly 50% of all NF1 cases represent newly introduced or spontaneous mutations without any familial history for NF1 (Huson and Hughes, 1994). The very diverse NF1 mutational spectrum ranges from small nonsense and missense mutations over insertions, small deletions and inversions to translocations, whole gene deletions and duplications (Messiaen et al., 2001). To date no consistent genotype-phenotype correlation has been found in patients in whom the disease mutation has been identified. The exception to this is the earlier onset of neurofibromas, occurrence of dysmorphic facial features and intellectual impairment in a subset of patients with NF1 with microdeletions that involve the entire NF1 gene and considerable flanking DNA (Kayes et al., 1994; Tonsgard et al., 1997). Patients with this NF1 microdeletions also tend to have a substantially higher life time risk for developing MPNSTs (De Raedt et al., 2003).

3 NF1 gene (product) expression

3.1 NF1 mRNA

The 11-13 kb NF1 transcript is ubiquitously expressed and has been detected in numerous human (brain, kidney, spleen, lung, muscle, neuroblastoma, melanoma, skin fibroblasts, epidermal melanocytes) and mouse (brain, kidney, B16 melanoma cells) tissues and cell lines (Wallace et al., 1990; Buchberg et al., 1990; Nishi et al., 1991). Different alternatively spliced NF1 mRNA transcripts (see figure 4) are expressed in a developmental- and tissue-dependent manner. Type I and type II isoforms were characterized first. Type II NF1 mRNA (exon 23a) is the most widely expressed transcript and contains an additional 63 nucleotides (nt) which lengthens the GAP-related domain (GRD) of the protein product with 21 amino aa (Marchuk et al., 1991). Interestingly, expression of both isoforms has been observed to be associated with differentiation status of particular tissues (Nishi et al., 1991). Type I predominates in fetal brain and undifferentiated primitive neuroectodermal tumors while type II was predominantly expressed in adult brain and differentiated cell lines. In contrast, a higher type I / type II NF1 mRNA ratio was found in adult brain (Suzuki et al., 1991). Schwann cell differentiation could be induced by cAMP stimulation and was associated with predominant expression of NF1 type II mRNA (Gutmann et al., 1993). The type III isoform has an alternatively spliced exon 48a of 54 nt (coding for an additional 18 aa) at the 3' end of the NF1 transcript, downstream of the GRD. Together with NF1 mRNA type IV, which contains both exons 23a and 48a, the expression of the type III isoform was characterized in muscle (Gutmann et al., 1995; Skuse and Cappione, 1997). The isoform (exon 9br, also called 9a) contains an additional 30 nt coding for 10 aa. It has been suggested to play a role in differentiation and development of neural tissues of the central nervous system (Danglot et al., 1995; Geist and Gutmann, 1996). Several isoforms have been described that are not yet confirmed at the protein level. A putative amino-terminal isoform (termed N-isoform 11), about 2.7 kb in length, codes for a truncated protein of 593 aa and shares the first 574 amino-terminal residues of the original (type I) NF1 gene product, thereby encompassing the first 11 exons. A similar isoform of 551 aa whose amino-terminal portion is encoded by the first 10 exons is termed N-isoform 10 (Suzuki et al., 1995). Alternative splicing of exons 29 and 30 produces three isoforms and omits either one or both exons, introducing a premature stop codon that leads to a truncated protein. These isoforms have a tissue-specific expression pattern with the exon 29 skipped transcript being expressed only in brain (Park et al., 1998). An amino-terminal isoform, termed NF1-10a-2, has an insertion of 45 nt between exon 10a and 10b and showed very low expression in the majority of human tissues. Amino acid sequence and motif analysis revealed that the insert coded for a transmembrane segment (Kaufmann et al., 2002).

3.2 Neurofibromin

Being an ubiquitously expressed protein, the levels of the NF1 gene product neurofibromin vary in different tissues. The protein was identified in all parts of the brain, especially in large projection neurons and Purkinje cells (Nordlund et al., 1993). Also strong expression was observed in Schwann cells of peripheral nerves (Nakamura et al., 1994). It was also located to keratinocytes and melanocytes in developing rat and human skin (Malhotra and Ratner, 1994). Only low levels were detected in cardiac tissues and no neurofibromin was observed in rat skeletal muscle, lung and kidney. The NF1 protein has a predicted molecular mass of 327 kDa based on the full length size of 2818 aa (Wallace et al., 1990; Marchuk et al., 1991). In gel the apparent size of neurofibromin seems to be around 220-250 kDa (Bollag et al., 1991; Gutmann et al., 1991), probably due to specific protein folding during gel eletrophoresis. There is no evidence of glycosylation or processing of the full length protein (Gutmann et al., 1993). The major functional domain in neurofibromin is a central region of about 360 aa (exons 20-27a) (see Figure 4) showing marked homology to the catalytic domain of mammalian GTPase-activating protein (GAP) (Xu et al., 1990) and yeast IRA1 and IRA2, and is called the GAP-related domain (GRD) which has already been well characterized (Scheffzek et al., 1998). Fahsold et al (2000) described a new functional domain upstream of GRD on the basis of an unusual clustering of missense mutations identified in a large cohorte of NF1 patients. This domain (comprising exons 11-17) seems to coincide with the cysteine / serine rich domain (CSRD) described by Izawa et al (1996). This CSRD comprised amino acids 543 – 909 in which three cysteine pairs (suggestive of ATP binding) and three potential cAMP-dependent protein kinase A (PKA) recognition sites (obviously phosphorylated by PKA) are present. There is reason to believe that a Sec14p-like domain is located downstream of GRD. This domain is homologous to the lipid-binding domain of the Saccharomyces cerevisiae phosphatidylinositol transfer protein Sec14p, suggesting a possible link between binding of lipids by these proteins and the regulation of Ras and Rho GTPases. However, structural divergence in the amino-terminal α-helical region of this domain in neurofibromin suggests that only the lipid-binding property may exist and that the phospholipid transfer activity is probably lacking (Aravind et al., 1999). Only recently a novel bipartite phospholipid-binding module was structurally discovered, composed of a Sec14p-homologous segment and a previously undetected pleckstrin homology (PH)-like domain, and showed phospholipid binding (D'Angelo et al., 2006). Further downstream of GRD towards the carboxy-terminal domain (CTD) and based on a highly expressed splice variant lacking exon 43, a functionally active bipartite nuclear localization signal (NLS) was identified which was necesseray and sufficient to translocate a NF1-GFP fusion protein (containing the carboxy-terminal part of neurofibromin) to the nucleus (Vandenbroucke et al., 2004).

4 Gene function, molecular interactions and subcellular localization

4.1 NF1 gene function

The identification of oncogenes and tumor suppressor genes has made it clear that these genetic components are involved in tumorigenesis (Diamandis, 1992). The genetic "two hit" model of tumor suppressor genes has been described by Knudson (1971) and has been proposed as one of the several mechanism for tumorigenesis. In this model the inactivation of one or both alleles of a tumor suppressor gene is supposed to be involved in the development of a particular type of malignancy. NF1 patients have multiple benign tumors that can predispose to malignancy. The observation that the central domain of neurofibromin shows sequence homology to the catalytic domain of GAP and yeast IRA1 and IRA2 which can downregulate $p21^{Ras}$ activity (Xu et al,1990), suggested that the NF1 gene / neurofibromin is a tumor suppressor gene / protein. It is thought to play a crucial role in cAMP-dependent PKA and $p21^{Ras}$-associated signaling pathways. Neurofibromin acts as a negative regulator of the latter pathway by accelerating the intrinsic GTPase activity of $p21^{Ras}$, which results in an increased switch of GTP-

bound, active p21^{Ras} into a GDP-bound, inactive form (Bollag and McCormick, 1991; see also Figure 2). The regulation of this molecular switch plays a critical role in the control of cellular growth and differentiation. Hyperactivation, due to activating mutations or distorted regulation of p21^{Ras} activity, can lead to aberrant signaling for cell proliferation and are involved in the genesis of many human (malignant) tumors (Bos, 1989). Several studies have shown that neurofibromin may regulate the cAMP / PKA pathway (Guo et al., 2000; Tong et al., 2002; see Figure 6). In Drosophila *melanogaster* and mice it was shown that neurofibromin regulates the activity of AC. More critical clues to the biological function of neurofibromin are provided by studies on the subcellular localization, and molecular associations and interaction partners.

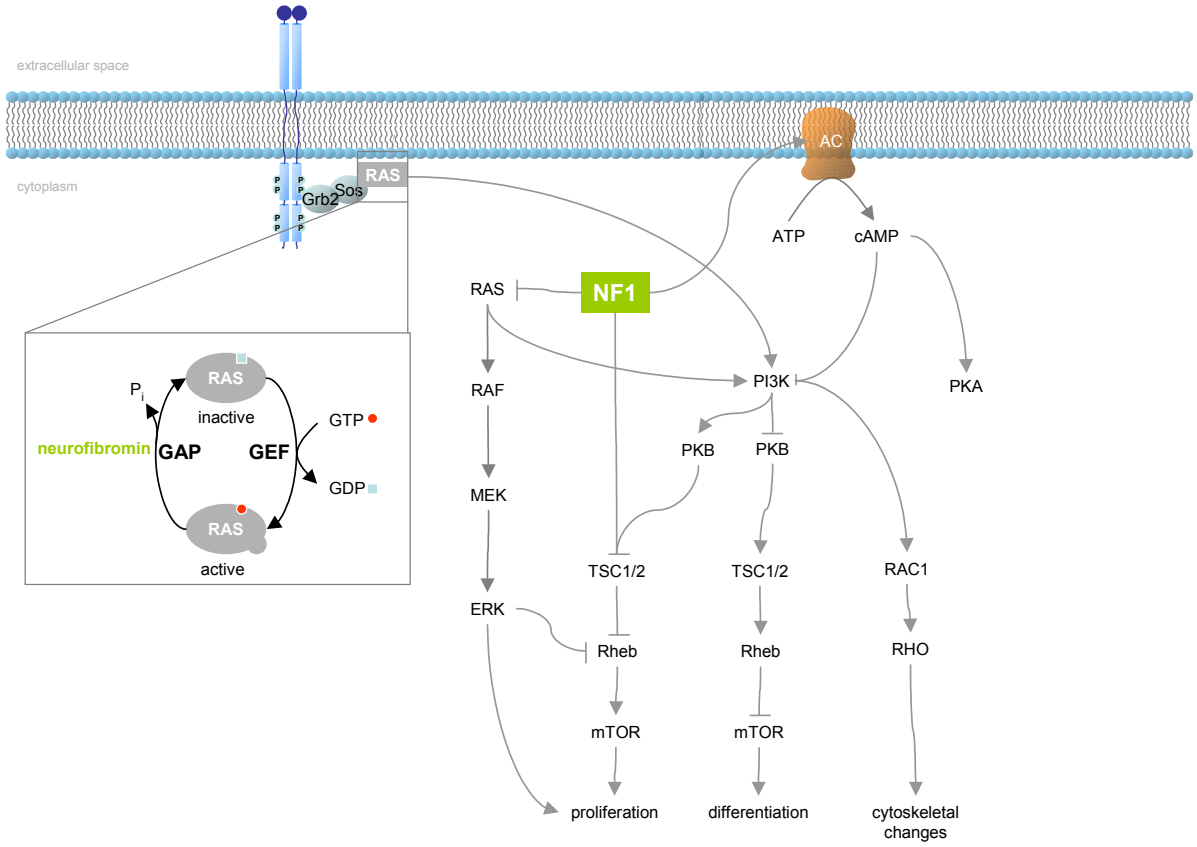

Figure 6. The interactions of neurofibromin with different functional signal transduction pathways. In addition to its function as a negative regulator of p21^{Ras} (Ras-GAP), the NF1 gene product may also be acquired in the regulation of cAMP- and PKA mediated gene transcription (Guo et al., 2000) and cytoskeletal changes. Recently, the NF1 tumor suppressor has been shown to be a critical regulator of the TSC2 – mTOR signal transduction pathway regulating cellular proliferation and differentiation (Johanessen et al., 2005).

4.2 Molecular interactions and subcellular localization

Neurofibromin has been shown to associate with the three major cytoskeletal systems. The first discovered association was with the microtubule cytoskeleton. The association with tubulin was shown to inhibit the p21^{Ras}-GAP activity of neurofibromin. The critical residues for this interaction reside in the GRD (Gregory et al., 1993; Bollag et al., 1993; Xu et al., 1997). Partial clues to its putative function have also been given by the interaction of neurofibromin with components of the microfilament (actin) cytoskeleton. Li et al (2001) demonstrated in telencephalic neurons and astroblasts that a differential subcellular targeting of neurofibromin towards the actin or

microtubule cytoskeleton depends on the developmental status. Heterozygosity at the NF1 gene locus was shown to result in aberrant cell attachment and increased cell motility due to actin cytoskeletal abnormalities (Gutmann et al., 2001). Similarily in a recent study, Ozawa et al (2005) showed that RNA interference-induced NF1 gene silencing enhanced cell motility and dynamically regulated actin cytoskeletal reorganization. An association of neurofibromin with the intermediate type filamentous (keratin) cytoskeleton in cultured differentiating epidermal keratinocytes was suggested to control the organization of the cytoskeleton during the formation of desmosomal cellular contacts (Koivunen et al., 2000; Koivunen et al., 2002). Malminen et al (2002) also demonstrated that neurofibromin, through its association with cytokeratin 14, may function in the regulation of epidermal histogenesis via the control of the keratin cytoskeleton during assembly of (hemi)desmosomes. Closely related to the cytoskeleton is the association of neurofibromin with the motor protein kinesin-1, which has been shown to transport protein complexes, organelles and mRNA to specific destinations in a ATP- and microtubular dependent fashion. This association, identified by purification of a particulate core-complex that also contained neurofibromin 2 (merlin), suggests a role for both tumor suppressor proteins in microtubule-mediated intracellular signal transduction pathways and vesicular cargo transport (Hakimi et al., 2002).

Using yeast-two-hybrid analysis Hsueh et al (2001) identified syndecan-2 as cellular interacting protein of neurofibromin. Syndecan-2 is member of the transmembrane family of heparan sulfate proteoglycans on the cell surface (Carey, 1997). They suggested that this interaction might be a potential mechanism for the membrane association of neurofibromin to specialized domains in the plasma membrane. This association might contribute to synaptic $p21^{Ras}$-GAP activity or function in an adhesion-signaling complex at the cell surface.

The protein N^G,N^G-dimethylarginine dimethylaminohydrolase (DDAH) that is known as a cellular nitrogen oxide (NO) / nitrogen oxide synthase (NOS) regulator has been identified as neurofibromin-associating protein. The DDAH-binding regions of neurofibromin were located in the CTD and the CSRD. They were found to coincidently contain the sites for PKA phosphorylation. DDAH increased PKA phosphorylation of native neurofibromin suggesting that PKA accessibility of neurofibromin is regulated via DDAH interaction and might modulate both the cAMP / PKA-related signals and the Ras-associated signals of neurofibromin (Tokuo et al., 2001).

The same group discovered a novel cellular neurofibromin-associating protein, 14-3-3, which belongs to a highly conserved family of proteins that regulate intracellular signal transduction events. 14-3-3 functions as a chaperone by regulating catalytic activities, protein-protein interactions and subcellular localization of bound proteins (Feng et al., 2004). The interaction is mainly directed to the CTD of neurofibromin. They also showed that PKA-induced phosphorylation of neurofibromin enhanced the interaction with 14-3-3 and suppressed the binding activity of neurofibromin to $p21^{Ras}$-GTP.

Four potential caveolin binding domains have been found in the primary structure of neurofibromin and that this interaction modulates $p21^{Ras}$, Akt and focal adhesion kinase (Boyanapalli et al., 2006). Caveolins have been shown to bind a growing number of signaling molecules within cholesterol-rich lipid raft microdomains (Sargiacomo et al., 1995).

If we overview the subcellular localization of neurofibromin based on known molecular interactions, protein motifs and domains, it is obvious that neurofibromin is located to a variety of subcellular compartments in different cell types. Primarily neurofibromin is found in membrane and cytosolic (particulate) fractions, while a few studies (together with the identification of a bipartite NLS in exon 43) have shown a nuclear localization (Daston et al., 1992; Golubic et al., 1992; Koivunen et al., 2000; Vandenbroucke et al., 2004). An association has been described with the plasma membrane (Boyer et al., 1994; Malhotra and Ratner, 1994), the smooth endoplasmic reticulum (SER) in neurons (Nordlund et al., 1993) and mitochondria (Roudebush et al., 1997).

5 Role of the micro-environment in NF1 etiopathogenesis

Haploinsufficiency of NF1 (=a situation in which the total level of a gene product produced by the cell is about half of the normal level and that is not sufficient to permit the cell to function normally) in the tumor microenvironment is becoming increasingly appreciated to have a role in tumor progression. In mouse models, it has been shown that a deletion of both copies of the Nf1 gene ($Nf1^{-/-}$) in Schwann cells (using a Krox 20-Cre promotor to induce Schwann-cell specific somatic inactivation of Nf1) combined with Nf1 heterozygosity ($Nf1^{+/-}$) of the tumor microenvironment (fibroblasts, endothelial cells, perineurial cells, mast cells) promoted neurofibroma formation. This system could be used as a model for the study of heterotypic cell interactions (Zhu et al., 2002). The Schwann cell was confirmed to be the cell of origin in the development of (plexiform) neurofibromas. In NF1 patients and mice with a germline knockout of one Nf1 allele, all of the cells in the body are heterozygous for NF1, raising the question of whether heterozygous neighbouring cells can promote tumor growth more efficiently than wild-type neighbouring cells. Implicit is the possibility that heterozygous inactivation of Nf1 has functional consequences (haploinsufficiency). It was shown that homozygous Nf1 mutant ($Nf1^{-/-}$) Schwann cells secrete large amounts of KitL (Kit ligand or SCF or mast cell growth factor), stimulating mast cell migration and infiltration in the neurofibroma. These $Nf1^{+/-}$ mast cells were shown to be hypermotile in response to KitL and their increased cell migration was linked to hyperactivation of the Ras-class IA-PI3K-Rac2 pathway (Yang et al., 2003). Increased activation of this pathway was also shown to be responsible for increased proliferation and survival of $Nf1^{+/-}$ mast cells (Ingram et al., 2001).

Whether this model for heterotypic cell interactions is applicable on other cardinal features of NF1, like the hyperpigmentary CALMs is currently unknown but is an interesting path for further investigation. Initial studies have seen that cultured dermal fibroblasts of normal and CALM skin of NF1 patients secrete higher levels of SCF and HGF, and that dermal mast cell numbers are increased (Okazaki et al., 2003; see **chapter 1 – article II**; see General discussion and future perspectives p.126).

The melanocyte and Neurofibromatosis type 1: pigmentary defects

Article I

Pigment cell-related manifestations in neurofibromatosis type 1: an overview.

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In this review we give a detailed overview of the pigmentary defects in Neurofibromatosis type 1 (NF1). The hyperpigmentary anomalies that are observed and described as cardinal clinical features of NF1 can be divided in two major groups based on the involvment of the cutis: (i) the cutaneous hyperpigmentary defects (including café-au-lait macules [CALM] and intertriginous ephelides) and (ii) the non-cutaneous defects (including iris Lisch nodules). In overviewing these hyperpigmentary anomalies it becomes clear that the melanocyte is playing a key role in the etiopathology of these lesions. We address several hypotheses of CALM etiopathogenesis and finally draw attention to a readily seen phenomenon of an increased incidence of melanoma in NF1 patients: a mere coincidence or is more going on?

Review
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Pigment cell-related manifestations in neurofibromatosis type 1: an overview

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Summary

Neurofibromatosis type 1 (NF1) is an autosomal dominant neurocutaneous disorder, affecting approximately 1 in 3500 individuals. The most commonly seen tumors in NF1 patients are the (sub)cutaneous neurofibromas. However, individuals with NF1 typically present in childhood with well-defined pigmentary defects, including café-au-lait macules (CALMs), intertriginous freckling and iris Lisch nodules. NF1 is considered a neurocristopathy, primarily affecting tissues derived from the neural crest. Since the pigment producing melanocyte originates in the neural crest, the presence of (hyper)pigmentary lesions in the NF1 phenotype because of changes in melanocyte cell growth and differentiation is to be expected. We want to discuss the pigmentary cutaneous manifestations of NF1 represented by CALMs and intertriginous freckles and the pigmentary non-cutaneous manifestations represented by iris Lisch nodules. Several hypotheses have been suggested in explaining the poorly understood etiopathogenesis of CALMs. Whether other pigmentary manifestations might share similar etiopathogenic mechanisms remains obscure. Additional attention will be drawn to a readily seen phenomenon in NF1: hyperpigmentation overlying (plexiform) neurofibromas, which could suggest common etiopathogenetic-environmental cues or mechanisms underlying CALMs and neurofibromas. Finally, we want to address the relationship between malignant melanoma and NF1.

Key words: melanocyte/neurofibromatosis type 1/café-au-lait macules/lisch nodules/freckling/melanoma

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Introduction

Neurofibromatosis type 1 (NF1) is one of the most common autosomal dominantly transmitted neurocutaneous disorders known to man, with an estimated prevalence of about 1 in 3500 individuals (Poyhonen et al., 1997) and affecting the development and growth control of a variety of tissues (Huson & Hughes, 1994). The disease was first described by von Recklinghausen (1882) and is therefore also known as von Recklinghausen disease. In 1988, during the National Institutes of Health (NIH) Consensus Development Conference, the diagnostic criteria for NF1 were set, which were later re-evaluated and recommended for continued use without modifications (Gutmann et al., 1997; Table 1). The most common tumors seen in individuals with NF1 are the (sub)cutaneous and (diffuse) plexiform neurofibromas (Gutmann et al., 1997). Other major primary features are the pigmentary defects of cutaneous [café-au-lait macules (CALMs), axillary and inguinal freckling] and non-cutaneous nature (iris Lisch nodules), optic pathway gliomas and osseous lesions (e.g. dysplastic vertebrae). Common clinicopathologic consequences of the primary features and complications thereof include, e.g. learning disabilities, short stature, scoliosis, epilepsy, intracranial tumors and renal artery stenosis.

NF1 is known for its highly variable clinical expression spectrum and approximately 30–50% of all patients lack a family history of the disease, representing new mutations of the *NF1* gene. Considering the prevalence of NF1, this represents one of the highest mutation rates of a human single-gene locus (Huson & Hughes, 1994). The responsible *NF1* gene was identified and sequenced in 1990 (Cawthon et al., 1990; Viskochil et al., 1990), is localized to chromosome 17q11.2 and spans approximately 335 kb of genomic DNA (Li et al., 1995) with a transcript of 11–13 kb long (Buchberg et al., 1990) and an open reading frame of 8454 nucleotides (Figure 1). This results in a large, full-length protein of 2818 amino acids, called neurofibromin (Marchuk et al., 1991). Affected individuals are heterozygous for an *NF1* mutation and the types of mutation include nonsense (premature termination of growing polypeptide) and missense mutations, insertions (frameshifts), small deletions, inversions, translocations, whole gene deletions and mutations in promoter and enhancer elements (Shen et al., 1996). Until now, no definite genotype-phe-

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Table 1. NIH diagnostic criteria for neurofibromatosis type 1 (NF1)

NF1 is present in a patient who has two or more of the following signs

- Six or more café-au –lait macules (greatest diameter >5 mm in prepubertal patients or >15 mm in postpubertal patients)
- Two or more neurofibromas or 1 or more plexiform neurofibromas
- Axillary or inguinal freckling
- Optic pathway glioma
- Two or more Lisch nodules (iris hamartomas)
- Distinctive osseous lesion (sphenoid wing dysplasia, thinning of cortex of long bones with or without pseudarthritis)
- First degree relative with NF1 by the criteria above

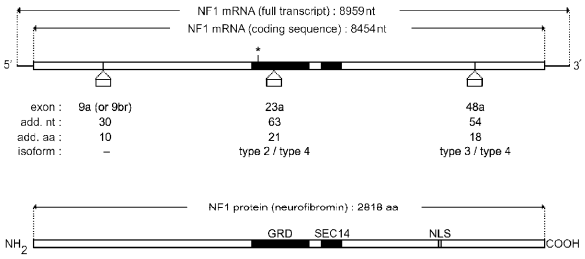

Figure 1. Schematic representation of neurofibromin gene organization, well-known isoforms and functional or structural domains. Add. nt: additional nucleotides; add. aa: additional amino acids.

notype correlations have been found, except for a more or less distinct severe phenotype associated with deletions of the entire *NF1* gene locus including the three embedded genes EVI2A, EVI2B and OMGP (intron 27) and sequences flanking the *NF1* gene (North, 2000). Recently, an elevated risk for the development of malignant peripheral nerve sheath tumors (MPNSTs) was described in *NF1* microdeletion patients (De Raedt et al., 2003).

A small portion of the central domain of neurofibromin shows sequence similarity to the family of GTPase–activating proteins (GAPs) (Buchberg et al., 1990) and is, therefore, called GAP-related domain (GRD) (Figure 1). GAPs convert Ras from its active GTP-bound form to its inactive GDP-bound form, down-regulating Ras activity and cell proliferation. The *NF1* gene is, therefore, considered as a tumor-suppressor gene. Indeed, mutations in both *NF1* alleles [loss of heterozygosity (LOH)] have been detected in malignant tumors associated with NF1 and in benign neurofibromas (Arun & Gutmann, 2004; Legius et al., 1993; Lynch & Gutmann, 2002; Serra et al., 2001). In cases of NF1 tumors where no LOH is detected, mRNA editing at a post-transcriptional level could account for the tumorigenesis (Cappione et al., 1997; Methany et al., 1995). However, it is not yet known how *NF1* gene mutations can cause many of the non-tumor related and hyperpigmentary manifestations such as café-au-lait spots, intertriginous freckling or iris Lisch nodules. In this light one might suspect neurofibromin functions unrelated to its GRD, as suggested by the detection of an isoform lacking this domain (Suzuki et al., 1992).

Recently, a high number of alternatively spliced exons have been identified and one of the most common (exon 23a) incorporates 21 amino acids into GRD of neurofibromin, producing two isoforms with a differential expression in certain tissues and under certain conditions. There is, however, still a lot to be learned on the predominance of some of the less abundant transcripts in several tissues (Andersen et al., 1993a,b; Suzuki et al., 1991; Vandenbroucke et al., 2002a,b) (Figure 1). The *NF1* gene (product) is widely expressed in a variety of human and rat tissues, including keratinocytes and melanocytes in developing rat and human skin (Malhotra & Ratner, 1994). The vast majority of NF1 gene mutations do not disrupt the GRD, which calls for speculation concerning other biological activities of neurofibromin. Six potential serine/threonine cAMP-dependent protein kinase (PKA) recognition sites and one potential tyrosine phosphorylation site have been recognized in neurofibromin, suggesting that the protein might be regulated by kinases (Fahsold et al., 2000; Lakkis & Tennekoon, 2000). Recently Vandenbroucke et al. (2004) identified a functional nuclear localization signal (NLS) in exon 43 of the *NF1* gene, showing that a highly expressed splice variant lacking this NLS controlled localization and hence the function of neurofibromin. In addition it has been demonstrated in *Drosophila melanogaster* and in mice that neurofibromin modulates the activity of adenylyl cyclase (AC) (Guo et al., 2000; Tong et al., 2002). This shows that neurofibromin is able to regulate the activity of two major signal transduction pathways (G-protein coupled receptor signaling pathway and tyrosine kinase receptor signaling pathway), influencing proliferation as well as differentiation in several cell types. Recent data suggest that regulation of AC activity by neurofibromin might be important in NF1 manifestations requiring LOH, whereas the Ras-GAP activity of neurofibromin could participate in NF1 symptoms manifest in heterozygosity such as learning deficits (Costa & Silva, 2003).

Mouse models could help to unravel the etiopathogenesis of the benign pigment-cell related manifestations of NF1. Mice homozygous for a deletion in the *Nf1* gene die in utero because of a hyperproliferation of endocardial cushions (precursors of the cardiac valves) in the heart. These endocardial cushions form through an endocardial-mesenchymal transformation, for which neurofibromin is required (Lakkis & Epstein, 1998; Lakkis et al., 1999). However, $Nf1^{-/-}$ chimeric mouse models have been established for neurofibromas, MPNSTs, myeloid leukemia and learning deficits but not for the pigmentation defects (Dasgupta & Gutmann, 2003; McClatchey & Cichowski, 2001). $Nf1^{+/-}$ mice

have been shown to have abnormal responses to skin wounding (Atit et al., 1999) and develop skin pigmentation abnormalities and papillomas upon challenge with either a skin cancer initiator or a tumor promotor (Atit et al., 2000). Our knowledge concerning the etiopathogenesis of NF1 is under full expansion. The mechanisms underlying the etiology and development of the characteristic pigmentary defects seen in NF1 are still very obscure. Therefore, we would like to give an overview of what is currently known about pigment cell-related manifestations in NF1.

Pigmentary cutaneous defects in NF1

Café-au-lait macules

The largest and best known pigmentary defects in NF1 patients are the CALMs, which are in general typically ovoid-shaped, sharply bordered, uniformly colored, benign, hyperpigmentary patches of the skin (Figure 2A). The occurrence of CALMs is not restricted to certain (segmental) regions of the body, but appears to be stochastically dispersed in the skin (Kestler & Haschka, 1998), even crossing the body midline. The CALMs are seen in most NF1 patients at or shortly after birth (Huson & Hughes, 1994; Huson et al., 1989). Based on data from the National Neurofibromatosis Foundation International Database, the prevalence is around 95–100% in children younger than 1 yr and remains this high throughout life. These hyperpigmentary spots are clinically very striking features of NF1, but their etiopathogenesis is still poorly understood. However, the CALMs are not pathognomonic for NF1 as they also have been reported clinically in other disorders like McCune–Albright syndrome, tuberous sclerosis or Leopard syndrome (Tekin et al., 2001). These macules are not even unique clinical features of certain Mendelian disorders as they are also observed in the normal population albeit at a lower incidence (around 10%).

Early histological studies on the pigmentary CALMs of NF1 patients have mainly focused on melanocyte densities in the epidermal basal membrane. Some inconsistencies arose, when certain authors demonstrated a significant increase in melanocyte density in CALMs compared with the normally pigmented skin of the same patient (Johnson & Charneco, 1970), while others only observed this difference in a few cases (Benedict et al., 1968). Frenk & Marazzi (1984) analyzed the ratio of melanocytes and keratinocytes in normal and CALM skin of NF1 patients compared with skin of healthy individuals. They observed a decrease in the keratinocyte/melanocyte ratio in NF1 epidermis, being slightly more pronounced in CALM skin but also manifest in normally pigmented skin, which could be interpreted as an aberrant organization of the epidermis of NF1 patients in light of the keratinocyte/melanocyte interaction. In the late 1950s ultrastructural studies of CALMs of NF1 patients revealed the presence of giant pigment granules in epidermal melanocytes and keratinocytes. Jimbow et al. (1973) distinguished several ultrastructural subunits and defined three subtypes of giant pigment granules in NF1 CALM epidermis, which could reflect a developmental sequence. Like normal melanosomes these giant pigment granules showed a surrounding membrane, tyrosinase activity and electron dense material, and were, therefore, referred to as macromelanosomes. The NF1 CALM melanocytes showed an increased number of melanin macroglobules (MMGs) (Martuza et al., 1985). This was also confirmed on in vitro melanocyte cultures derived from unaffected and NF1 CALM skin and from healthy donor skin, where more MMGs were found in NF1 melanocytes with the highest numbers in NF1 CALM melanocytes (Kaufmann

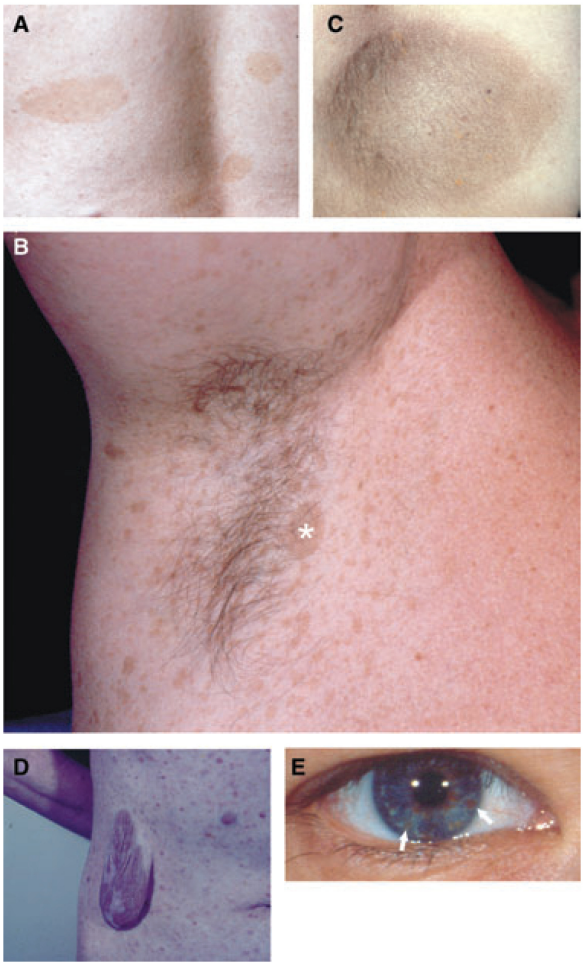

Figure 2. (A) Multiple café-au-lait spots on the trunk of patient with NF1. (B) The right axilla of an NF1 patient showing axillary freckling, a café-au-lait spot (asterisk). (C) A hyperpigmented plexiform neurofibroma in a NF1 patient. Mark the mild hypertrichosis. (D) A hyperpigmented pedunculated plexiform neurofibroma on the trunk. (E) Lisch nodules (arrows) can sometimes be macroscopically evident.

et al., 1989). These MMGs are not specific for NF1 and have been seen in numerous other diseases and in normal skin (Jimbow & Horikoshi, 1982). Two possible mechanisms for the formation of these macromelanosomes have been suggested: they can be formed by a disturbed melanin production or by autophagocytosis of melanosomes (Jimbow & Horikoshi, 1982). Fusion of autophagosomes (accumulating melanosomes) and lysosomes is now considered to be the most likely origin of the macromelanosomes and their morphologic and enzymatic properties (e.g. acid phosphatase) are similar to those of lysosomes (Nakagawa et al., 1984).

Cell culture studies have shown that the NF1 gene defect affects melanogenesis in epidermal melanocytes of NF1 patients (Kaufmann et al., 1991), whereas Ras-GTP levels (indicative for the proliferation status of the cell) of NF1 derived melanocytes were comparable with those of healthy donor normal skin. Apparently, the difference seen in melanogenesis between healthy control and NF1 melanocytes could not be ascribed to a dysregulative Ras-activity (Griesser et al., 1995). Using in vivo immunohistochemistry no reduction in the levels of neurofibromin could be detected in NF1 café-au-lait and normal skin and levels were similar to those seen in healthy donors (Malhotra & Ratner, 1994). However, in melanocyte cultures derived from NF1 patients, an overall distinct decrease in neurofibromin expression was detected by immunoprecipitation and western blotting, possibly explained by an unequal expression of the NF1 alleles (Griesser et al., 1995). In NF1 CALM melanocytes on the other hand, variable expression of neurofibromin has been reported with the higher neurofibromin expression coinciding with an increased dendricity. It was shown that the neurofibromin level can vary in vitro over a wide range by using different culture conditions. Phorbol-12-myristate-13-acetate (PMA), basic fibroblast growth factor (bFGF) or stem cell factor (SCF), added to the culture medium of melanocytes obtained from healthy donors, increased the neurofibromin protein level. This was, however, not accompanied by a parallel increase in NF1 mRNA level suggesting a post-translational regulation of neurofibromin (Griesser et al., 1997). NF1 CALM melanocytes in culture medium, supplemented with the proliferation promotor PMA and the differentiation inducers choleratoxin (CT) and 3-isobuthyl-1-methyl-xanthine (IBMX), displayed a diminished proliferation, a higher melanin content, melanin synthesis rate and an aberrant morphology in comparison with normal healthy control melanocytes (Kaufmann et al., 1991). Evidence for an altered degradation rate of neurofibromin was also established in NF1 CALM melanocytes. Here, stimulation with PMA resulted in less increase in neurofibromin half-life compared with NF1 and healthy donor normal skin melanocytes (Kaufmann et al., 1999a). Neurofibromin, which is susceptible to lysosomal degradation through the presence of a special amino acid motif in its GRD, undergoes phosphorylation under PMA or bFGF stimulated culture conditions, thereby protecting it against lysosomal degradation (Kaufmann et al., 1999b). The possible role of neurofibromin in melanocyte differentiation was further elaborated by the transient co-expression in a melanoma cell line of rat neurofibromin cDNA and a fusion gene, consisting of the tyrosinase promotor region cloned upstream of the firefly luciferase reporter gene. This showed that the neurofibromin-GRD increased the activation of the tyrosinase gene promoter (Suzuki et al., 1994). Moreover, the expression of tyrosinase-related protein-2 (TRP-2), which is expressed in migratory melanoblasts and precedes the expression of tyrosinase and tyrosinase-related protein-1 (TRP-1), is regulated by neurofibromin, suggesting that TRP-2 rather than tyrosinase could be the key protein under neurofibromin regulation during melanocyte differentiation (Suzuki et al., 1998).

In a study of Ishida & Jimbow (1987) a computed image analyzing system was used to study numerical and morphological changes of NF1 CALM melanocytes. They found a CALM coloration dependent effect on several cellular parameters when compared with the normally pigmented melanocyte population, with the light and brown macules showing a normal population of melanocytes with increase in whole cell area and perimeter; cytoplasm area, perimeter and diameter; and dendrite area, length and breadth. The dark brown macules, however, showed an increase in epidermal melanocyte number but a decrease in all parameters mentioned above. Haploinsufficiency of the NF1 gene has been shown to result in an increased variation in the number and length of dendrites in NF1 melanocytes. This variability can be restored by culturing NF1 melanocytes on substrates with parallel stripes of specific dimensions, forcing the dendrites to grow along these guides (Jungbauer et al., 2004). Melanocytic dendrites direct their migration during embryogenesis and consequently altered dendrite formation can disturb migration of melanocyte precursors and result in an unequal distribution of pigment cells and hyperpigmentation (Kemkemer et al., 2002).

Contrary to NF1 malignant tumors and benign neurofibromas, in which LOH has already been detected, both alleles of the NF1 gene were found in NF1 CALM melanocytes and their X-inactivation pattern pointed in the direction of a monoclonal origin of these cells (Eisenbarth et al., 1997). NF1-derived cell cultures, including NF1 CALM melanocytes, display an increased spontaneous chromosomal instability, primarily situated in the centromeric regions. Non-allelic modifying genes could, therefore, contribute to some of the benign NF1 symptoms such as neurofibromas or café-au-lait spots (Kehrer & Krone, 1994).

Alternative splicing in the NF1 gene could be important for differentiation of neural crest-derived cells and for tumorigenesis. It has been postulated that a switch of NF1 mRNA isoform type 1 to type 2 may be

involved in neuroectodermal differentiation (Nishi & Saya, 1991). The development of café-au-lait spots in NF1, characterized by an altered growth and differentiation state of the melanocytes, does not result from a switch of *NF1* gene transcript type 1 to type 2, the latter being characterized by an in-frame insertion of 63 bp within the GRD of exon 23a. The ratios of both types of mRNA are similar to those seen in healthy donors, with a predominance of type 2 transcript in most cell types, except in blood cells (Eisenbarth et al., 1995).

Many studies have suggested that aberrant expression of growth factors and their receptors may play a role in the hyperproliferation of NF1 tumor cells (Mashour et al., 1999). NF1-related tumors could result from the loss of NF1 activity in a subset of cells with subsequent recruitment of additional cells, which are hypersensitive to growth factors present in the lesion (Korf, 2001). Regarding the etiopathogenesis of NF1, it seems that alterations in growth factor expression, like nerve growth factor (Fabricant & Todaro, 1981), ciliary neurotrophic factor (Asai et al., 1991), insulin-like growth factor-2 (Hansson et al., 1988), hepatocyte growth factor (HGF) (Krasnoselsky et al., 1994; Rao et al., 1997), SCF (Ryan et al., 1994) and the angiogenic factor midkine (Mashour et al., 2001), may play an important role. Several hypotheses have been proposed to explain the etiopathogenesis of the hyperpigmentary CALMs in NF1 patients and SCF apparently is an important player. Analogous to reports on the SCF secretion of Schwann cells and proliferation increase of mast cells in neurofibromas (Viskochil, 2003; Yang et al., 2003), one recent study suggests that the mechanism of epidermal hyperpigmentation in NF1 CALM is associated with cytokines such as SCF and HGF (Imokawa, 2004). Dermal fibroblasts would, for a yet undetermined reason, produce increased amounts of soluble SCF and HGF, affecting the dermal mast cells and epidermal melanocytes and resulting in a hyperpigmentary CALM. ELISA on NF1 CALM fibroblast conditioned medium revealed elevated secretion of SCF and HGF in comparison with those of NF1 normal skin and healthy normal skin. This was not seen in NF1 CALM keratinocyte conditioned medium. In addition, increased expression of SCF and HGF mRNA was demonstrated by RT-PCR in cultured NF1 CALM fibroblasts (Okazaki et al., 2003).

The group of Wehrle–Haller studies the role of soluble and membrane-bound SCF on the migration and survival of melanocyte precursors. They observed that during embryological development neural crest cells partially dispersed from the migration staging area (MSA) via a ventromedial pathway, while a remaining subpopulation of neural crest-derived cells remained at the MSA, expressing the SCF receptor c-kit and TRP-2 mRNA (typical for melanocyte precursors) and nesting themselves in a region between the dermatome and (future) epidermis. In other words, melanocyte precursors need SCF for their lateral pathway migration and initial survival in the MSA, but their survival in the dermal mesenchymal area is determined by membrane-bound SCF. Their hypothesis suggested that local fluctuations of keratinocyte-derived membrane-bound SCF could account for the pigmentary defects seen in NF1, as it has been shown that adult melanocyte survival depends on this membrane-bound form (Grichnik et al., 1998; Wehrle-Haller et al., 2001).

Finally, one group ascribed the development of CALMs to an embryological migration problem of melanocyte precursors. It has been stated that NF1 haploinsufficiency causes a disruption in dendrite formation of melanocyte precursors, and hence their migration, leading to an unequal epidermal distribution of melanocytes and CALM formation (Kemkemer et al., 2002).

Intertriginous freckling

Freckles (ephelides) are small, light brown macules found on sun-exposed skin areas of fair subjects. On the other hand, intertriginous freckling is a well known hyperpigmentary cutaneous manifestation in NF1 and is thought to be pathognomonic for the disorder (Figure 2B). The color of the freckles is similar to CALMs, but they are smaller and in greater number than the latter. They are located to body regions (axillae, inguinal regions, apposing skin surface below the breasts in women, base of the neck, upper eyelids) where the skin is thought to be influenced by certain physical properties such as increased skin temperature, absence of light exposure and skin secretions like sweat. Even friction of the skin which is caused by tight clothing or itching caused by pruritus can be considered as mechanistical inducers in the appearance and development of freckles. Axillary freckles can be visible at birth in NF1 patients but more often they appear later in childhood with a prevalence of more than 80% by 4 yr of age (Friedman et al., 1999).

An interesting observation is that the distribution and appearance of these hyperpigmentary lesions can be influenced by body (skin) temperature. Kaufmann et al. (2001) described the distribution of dermal neurofibromas in NF1 and found a relation with the temperature pattern of the skin surface. In the case of neurofibromas a distribution along the surface of the trunk was apparent. They hypothesized that the mechanisms functionally inactivating the NF1 gene could be more active in skin areas with higher temperature (trunk) than in areas which were cooler (arms/legs), as was seen in cases of somatic mutagenesis. These temperature sensitive mechanisms could affect NF1 transcript and protein stability, mRNA editing or even DNA repair systems. In addition, it is described that temperature regulates melanin synthesis via tyrosinase activity in melanocytes (Kim et al., 2003) and that heat-treated melanocytes show an increased dendricity, a larger cell body and a higher tyrosinase activity (Nakazawa et al., 1998). Whether temperature sensitivity or other physical

properties of the skin could account for the development of CALMs and intertriginous freckles remains unclear. Riccardi even suggested that the mechanism of freckle and CALM development in NF1 patients could be different, based on the distribution patterns (CALMs random, freckles intertriginal) and influence of local factors (heat, UV light, salinity) (Riccardi, 1981).

To our knowledge the only study investigating differences between sun-exposed freckles (ephelides) in healthy persons and the freckling in NF1 was conducted in 2001 by the group of Amer et al. (2001). Clinically the two types of lesions can be differentiated via their site of appearance (sun-exposed vs. intertriginous areas), their size (larger in NF1), their color (darker brown in NF1), the skin-type of those affected (fair in ephelides) and response to UV-exposure or bleaching agent (no response in NF1). Light microscopic studies are similar in both types of freckling except for the elongation of rete ridges seen in NF1 freckles and CALMs. The most obvious differences could be found with transmission electron microscopy displaying an increased number of melanocytes in NF1 freckles and CALMs in addition to the giant melanin granules.

A limited number of cases of partial unilateral lentiginosis (PUL) associated with segmental neurofibromatosis (unilateral, contralateral and in one case bilateral) have been described (Allegue et al., 1989; Gerhards & Hamm, 1992; Lee et al., 1995; Piqué et al., 1995; Wong, 1997). Partial unilateral lentiginosis is a rare disorder of cutaneous pigmentation first described in 1904 which can appear without associated conditions. The association with CALMs, freckling, neurofibromas or Lisch nodules supported some authors to consider PUL as a forme fruste of NF1 (Thompson & Diehl, 1980). This is further demonstrated by the case of a girl with NF1 whose mother suffered from PUL with one CALM and unilateral Lisch nodules (Moss & Green, 1994). A somatic mutation affecting a single cell in early embryonic development is considered to be the cause for segmental neurofibromatosis. For PUL, which is also considered to be because of a mosaicism, no genomic alterations have been identified.

Hyperpigmentation overlying (plexiform) neurofibromas

Less than 1% of all neurofibromas (NF1 and non-NF1 related) registered in the Soft Tissue Registry of the Armed Forces Institute of Pathology between 1970 and 1996 are pigmented (Fetsch et al., 2000). Hyperpigmentation overlying (plexiform) neurofibromas should be differentiated from neurofibromas arising within a café-au-lait spot, which can be seen occasionally. Cutaneous hyperpigmentation can be the first indication of a deep plexiform neurofibroma. This can also indicate tumor aggressiveness or spinal cord involvement and warrants a close follow-up (Riccardi, 1980). The hyperpigmentation is sometimes accompanied by localized hypertrichosis (Ettl et al., 1996) and a biopsy may be necessary to differentiate it from Becker's nevus (Chapel et al., 1981; Mahe et al., 2001) (Figure 2C, D). It can also resemble a nevus spilus (Finley & Kolbusz, 1993; Paik et al., 1996).

It is unclear whether the melanin synthesis in neurofibromas is caused by pigment-synthesizing Schwann cells or by a co-existing melanocytic lesion (Jurecka et al., 1988). Electron microscopic study showed that neoplastic Schwann cells are capable of melanogenesis in some cases (Anderson & Robertson, 1979) and, moreover, there is evidence for the possibility of transition of differentiated Schwann cells to melanocytes in clonal cultures under the influence of growth factors. It was recently described that Schwann cells exposed to endothelin-3 can transdifferentiate into melanocytes through reversion to the stage of bipotent glial-melanocytic neural crest precursors (Dupin et al., 2003). Earlier publications showed that Schwann cell precursors could give rise to melanocytes following treatment with bFGF or 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) (Sherman et al., 1993). This again demonstrates the close relationship between Schwann cell-derived and melanocytic tumors.

Pigment-cell related non-cutaneous manifestations in NF1

Lisch nodules

One of the diagnostic criteria for NF1 is the presence of two or more Lisch nodules (iris hamartomas) (Riccardi, 1980). This type of ocular manifestation has been described for the first time at the beginning of the 20th century (Snell & Treacher Collins, 1903). Several years later other descriptions and a first histological analysis of these iris nodules were published (Fuchs, 1913; Waardenburg, 1918). Association of these nodules with NF1 was made in the 1930s (Goldstein & Wexler, 1930; Lisch, 1937; Sakurai, 1935). Based on Lisch's work these iris hamartomas were designated as Lisch nodules. In 1993, Ragge (1993) even proposed to rename these nodules into Sakurai–Lisch nodules, to give credit for the excellent and detailed work done by Sakurai 2 years before Lisch's publication.

Lisch nodules are well-defined, avascular, smooth, regular, dome-shaped elevations of the iris surface, having a yellow to brown color (Lewis & Riccardi, 1981) (Figure 2E). They can be clearly distinguished from iris naevi which show a more flattened appearance. The lesions occur mainly bilateral. They vary in number and size with nodule diameters going up to 2 mm, even being macroscopically visible (Ragge, 1993). The iris nodules have a characteristic appearance and they are mainly identified through slit-lamp photographic examination by an experienced ophthalmologist.

Because of the difficulty to directly investigate these Lisch nodules on a (sub)cellular and molecular level, mainly the prevalence in the NF1 population has been studied. Several studies have demonstrated that the

incidence ranges from 73 to 95% in NF1 (Huson et al., 1987; Lewis & Riccardi, 1981; Lubs et al., 1991; Otsuka, 2001; Zehavi et al., 1986). A meta-analysis study, combining and integrating prevalence data from six large studies, has shown that the prevalence of Lisch nodules gradually increases from birth (no nodules) to 50% in 5-yr-olds, 75% in 15-yr-olds and going up to 95–100% in adults over 30 yr old (Ragge, 1993). A clear positive correlation can be seen between the number of Lisch nodules, the severity of other cutaneous manifestations and the age of the patient (Korakawa & Makita, 1977; Lewis & Riccardi, 1981; Ragge, 1993; Zehavi et al., 1986). Based on these observations the presence of Lisch nodules are virtually pathognomonic for NF1 and, therefore, they can be a very helpful diagnostic marker for NF1, especially when one tries to diagnose NF1 in children or when problematic diagnostic cases are presented (Huson et al., 1987; Toonstra et al., 1987; Zehavi et al., 1986). Attention must be drawn to the fact that, if children with multiple CALMs are found to have Lisch nodules, they are diagnosed with NF1. However, children of NF1 patients having no CALMs and Lisch nodules at the age of 5 yr are excluded from NF1 diagnosis and did not inherit the mutated NF1 allele (Huson et al., 1987).

(Ultra)structural, histological and cellular studies of the Lisch nodules have mainly been done by light and electron microscopy on iridectomy or postmortem iris specimens (Perry & Font, 1982; Williamson et al., 1991). Histologically two lesional compartments can be found in these nodules: a superficial plaque of compact spindle-like cells, and an underlying stromal accumulation of similar but loosely organized cells, both being pigmented and according to the study of Perry and Font being melanocytes (Perry & Font, 1982; Williamson et al., 1991). The Lisch nodule melanocytes appear to be larger than the normal iris melanocytes (Korakawa & Makita, 1977). This is an interesting finding because it has been observed previously that the melanocytes residing in the basal layer of NF1 café-au-lait skin also show an enlarged cell body (Ishida & Jimbow, 1987). Immunohistochemical analysis of these cells showed a positive staining for vimentin and S-100 protein, indicative for neuroectodermal (neural crest) origin and differentiation, much like melanocytes in CALMs and Schwann cells in (cutaneous) neurofibromas (Lukacs et al., 1997). A recent histological and ultrastructural analysis of a Lisch nodule revealed three main cell types: pigmented cells, fibroblast-like cells and mast cells, which resembles the neurofibroma cell population. The pigmented cells display features of Schwann cells (pinocytotic vesicles along the inner surface of the plasma membrane and a discontinuous basement membrane) as well as of melanocytes (melanosomes and large melanosomal complexes), which is not surprising considering the common embryological neural crest background. The authors suggest a role for mast cells in both Lisch nodules and neurofibromas (Richetta et al., 2004).

Malignant melanoma in NF1: a mere coincidence?

Malignant tumors occur significantly more often (approximately four times higher) in NF1 patients compared with the general population matched for age, gender and time of follow-up (Zöller et al., 1997).

The simultaneous occurrence of malignant melanoma and NF1 was described for the first time in 1934 (Bjorneboe, 1934). In the last decades a number of clinical reports have described the occurrence of malignant melanoma in NF1 patients but a definite association has not yet been established (Gallino et al., 2000; Guillot & Delaunay, 1990; Karakayali et al., 1999; Mastrangelo et al., 1979; Miyauchi et al., 1988). Malignant melanoma in NF1 was seen at several sites including leptomeningeal, ocular, cutaneous and mucosal localization. Cutaneous melanoma has been reported in 0.1–5.4% of patients with NF1. As discussed above NF1 patients have an increased number of melanocytes in their café-au-lait spots as well as in their normal skin, suggesting a proliferative process of melanocytes in NF1. It is hypothesized that NF1 melanocytes are predisposed to malignant transformation because of alterations in the NF1 gene (Andersen et al., 1993a,b). Considering the relation between the NF1 gene mutation and several neural crest-related tumors (malignant peripheral nerve sheath tumors, pheochromocytoma, giant congenital melanocytic nevi, etc.), the association of NF1 and malignant melanoma is unlikely to be a mere coincidence (Duve & Rakoski, 1994). Inactivation of the NF1 gene was found in a number of melanoma cell lines (obtained from tumors in patients without NF1) having no impaired regulation of GTP-Ras (Johnson et al., 1993; Lu & Kerbel, 1994). Although it was first described that neither LOH nor mutations in the NF1-GRD could be detected by PCR and Southern Blot-based experiments in a total of 68 melanocytic lesions (Gomez et al., 1996), in recent years a number of cases of LOH of the NF1 gene have been described. Loss of heterozygosity of the NF1 gene was found in 67% of cases of desmoplastic malignant melanoma (compared with 5% LOH in common melanomas) (Gutzmer et al., 2000) and in one case of malignant melanoma of the anus in man with NF1 (Ishii et al., 2001).

Until now the only differences observed between cutaneous melanoma in NF1 and in a control population is the female predominance, the lower age at diagnosis and the higher Breslow thickness (Guillot et al., 2004). A possible explanation for this higher Breslow thickness might be a delay in diagnosis because of the difficulty of identifying the malignant melanoma amongst the various skin lesions and hyperpigmentations seen in NF1. In a number of cases the cutaneous melanoma developed in giant

congenital nevi (Brasfield & Das Gupta, 1972; Hope & Mulvihill, 1981), café-au-lait spots (Perkinson, 1957), sunburned skin, nevus spilus (Rütten & Goos, 1990) or in skin overlying a neurofibroma (Silverman et al., 1988).

In 1990, a rare case of cutaneous malignant melanoma with osteoid formation, confirmed by the picrosirius-polarization method and by transmission electron microscopy, in a patient with NF1 was reported (Pellegrini & Scalamogna, 1990). Neoplasms with neural crest origin, especially malignant peripheral nerve sheath tumors, frequently seen in NF1, have been known to display areas of metaplastic differentiation or elements such as cartilage or bone (Guccion & Enzinger, 1979; Payne, 1960; Woodruff, 1976; Woodruff et al., 1973). Osteoid and bone formation in malignant melanoma, however, is rare with only a few cases described in literature (Banerjee et al., 1998; Giele et al., 2003; Grunwald et al., 1985; Hoorweg et al., 1997; Moreno et al., 1986; Pellegrini & Scalamogna, 1990).

There is an increased incidence of uveal nevi as well as uveal malignant melanoma, the most common primary malignant intraocular tumor in adults, in NF1 but the association NF1-uveal malignant melanoma remains controversial. Choroidal melanoma (Bacin et al., 1993) seems to be more prevalent in NF1 than iris melanoma (Honavar et al., 2000). Uveal melanomas frequently arise from pre-existing pigmented uveal lesions (small pigmented patches resembling cutaneous CALM or brownish Lisch nodules) (Dereure et al., 1995). It has been suggested that mutations of the NF1 gene may occasionally play a role in the pathogenesis of uveal melanoma (Foster et al., 2003). Ocular malignant melanoma involving the conjunctiva seems to be much less frequent (To et al., 1989). As described in cutaneous melanoma and other malignant neoplasms seen in NF1, ocular malignant melanoma has a clear predominance in female NF1 patients.

Concluding remarks and future perspectives

Because of its direct accessibility the skin has always been one of the major diagnostic tools in NF1. However, research on NF1 skin manifestations has mainly focused on neurofibromas, while the etiopathogenesis of café-au-lait spots and freckling is still poorly understood. Over the last few years increasing attention has been made to the role of mast cells and associated growth factors in NF1 pathogenesis but clear cut evidence for their role in the pigmentation-related symptoms is still lacking. In this manuscript, we reviewed on a clinical, epidemiological, histological and, if possible, a molecular biological level, the pigment cell-related manifestations of NF1 with a major focus on café-au-lait spots, intertriginous freckling and Lisch nodules. Indeed, these are minor, non life-threatening features of a multisystemic tumor-predisposing genetic disorder, which could explain the lack of attention paid to them in recent literature. Their study could, however, provide links to the general NF1 pathogenesis and identify molecular pathways altered by NF1 gene mutations. In the future, this might be a powerful tool for the development of targeted NF1 therapeutics.

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Article II

Café-au-lait spots of Neurofibromatosis type 1 patients and healthy controls: hyperpigmentation of a different kind?

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Café-au-lait spots in neurofibromatosis type 1 and in healthy control individuals: hyperpigmentation of a different kind?

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Abstract Solitary café-au-lait spots are quite common in the general population but multiple café-au-lait macules (CALM) are often indicative of an underlying genetic disorder. The frequency of having more than five CALM is rare in normal individuals and is therefore considered as a cut-off for the diagnosis of neurofibromatosis type 1 (NF1). The etiopathogenesis of these macules is still very obscure. In this study we compared epidermal melanocyte and dermal mast cell numbers between four groups: control normal and control CALM skin, and NF1 normal and NF1 CALM skin and elaborated a possible role for stem cell factor (SCF) in CALM formation. The groups were analyzed by immunohistochemistry for numerical analysis of the melanocyte and mast cell population and by ELISA, western blot analysis and real-time quantitative PCR for further determination of the role of SCF. We found a significant increase in melanocyte density in NF1 CALM skin compared with the isolated CALM in control individuals. However, both groups displayed a similar increase in mast cell density. In addition, we found increased levels of soluble SCF in NF1 CALM and in NF1 normal fibroblast supernatant. We conclude that SCF is an important cytokine in NF1 skin, but that additional (growth) factors and/or genetic mechanisms are needed to induce NF1-specific CALM hyperpigmentation.

Keywords Café-au-lait spot · Neurofibromatosis type 1 · Hyperpigmentation · SCF · Mast cell

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Abbreviations NF1: neurofibromatosis type 1 · SCF: stem cell factor · CALM: café-au-lait macule

Introduction

Café-au-lait macules (CALM) are well circumscribed, uniformly light to dark brown macules averaging 2–5 cm in adults, but they may vary from 2 mm to more than 20 cm. They can be present at birth but they usually develop during early childhood and grow proportionately to body growth. Isolated CALM are a common finding in 10–20% of normal healthy young children. A study by Alper and Holmes [2] showed CALM in 0.3% of Caucasian newborns compared with 18% of black African-American newborns. CALM do not show any tendancy towards malignancy and do not require medical treatment. Cosmetic improvement can be achieved by laser treatment. Having six or more CALM is one of the seven cardinal criteria in diagnosing neurofibromatosis type 1 (NF1) [14], an autosomal dominant disorder affecting about 1 in 3,500 individuals [45]. The disease is fully penetrant but has a highly variable clinical expression. The gene is located on the long arm of chromosome 17 (17q11.2) and encodes a large protein of 2,818 amino acids called neurofibromin [7, 33, 55]. This protein has a tumor suppressor function since its GAP-related domain (GRD) mediates the conversion of the active Ras-GTP to the inactive GDP-form [6].

Early histological studies of CALM in healthy individuals showed an increased melanin content, a normal number of melanocytes and the occasional presence of giant melanosomes in both melanocytes and basal keratinocytes [42]. Cell culture studies have shown that the NF1 gene defect affects melanogenesis in NF1 melanocytes, with higher melanin content in NF1 compared with control melanocytes. The largest amount of melanin is found in NF1 CALM melanocytes [26]. In addition, in vitro melanocyte cultures derived from NF1

unaffected and CALM skin and from healthy donor skin showed an increased number of macromelanosomes in NF1 melanocytes with the highest numbers in NF1 CALM melanocytes [21, 22, 25].

Many studies have suggested that aberrant expression of growth factors and their receptors may play a role in the hyperproliferation of NF1 tumor cells [3, 16, 46]. It was recently reported in mice that stem cell factor (SCF) secretion by $Nf1^{-/-}$ Schwann cells increased proliferation of mast cells and was important in the formation of neurofibromas [54, 60]. In addition an analogous cytokine-related mechanism has been described for NF1 CALM [18, 40]. Other groups suggest that local fluctuations of keratinocyte-derived membrane-bound SCF could be more important [12, 58]. Recently the development of CALM has also been ascribed to an embryological migration problem of melanocyte precursors. It has been stated that NF1 haploinsufficiency causes a disruption in dendrite formation of melanocyte precursors, and hence their migration, leading to an unequal epidermal distribution of melanocytes and CALM formation [28].

It is clear that the etiopathogenesis of CALM in NF1 patients, and also in control individuals, is still under discussion. In this study we examined melanocyte and mast cell numbers in control normal and CALM skin, and NF1 normal and CALM skin, and tried to link these results to a possible influence of the growth factor SCF.

Materials and methods

Patient selection

NF1 patients were selected via the Department of Dermatology of Ghent University Hospital and met the clinical diagnostic NIH criteria for NF1. In a number of patients the diagnosis was confirmed on a molecular genetic level through NF1 mutation analysis. Patient profiles of control and NF1 patients are described in Table 1. The collection of skin biopsies and blood plasma was performed after written informed consent and guided by the institutional ethics committee.

Skin biopsies and cell culture

Five-millimeter punch biopsies were obtained from normal and CALM skin of control patients and normal and CALM skin of NF1 patients after local anesthesia with a 2% lidocain solution without epinephrine. Some biopsies were formalin-fixed for immunohistochemical analysis, while other biopsies were brought in 15 mL of 10% Raid solution (mixture of DMEM, fetal calf serum [FCS] and Raid [PBS, fungizone, gentamycine]) and kept overnight at 4°C before further processing for cell culture. After a PBS washing step, biopsies were incubated overnight in 10 mL of 10% dispase at 4°C for enzymatic separation of the epidermal sheet from the underlying dermis.

Primary epidermal melanocyte cultures were established as described previously [37, 49]. Briefly, melanocytes were cultured in Ham's F10 medium (Gibco, Invitrogen Ltd, Paisley, UK) supplemented with 2.5% FCS, 1% Ultroser G (Biosepra SA, France), 5 ng/mL basic fibroblast growth factor (bFGF), 10 ng/mL endothelin-1 (ET-1), 0.33 nM cholera toxin (CT), 33 μM isobutyl-methyl-xanthine (IBMX), 5.3 nM 12-tetradecanoyl phorbol 13-acetate (TPA) and 20 ng/mL SCF. Seventy-two hours before cell harvesting, melanocytes were maintained in SCF-depleted medium to minimize the effects of this growth factor. Human fibroblasts were established as explants from dermal portions of skin biopsies and cultured in OptiMEM I (Gibco, Invitrogen Ltd, Paisley, UK) supplemented with 2% Ultroser G and 1% Glutamine. Human keratinocytes were cultured according to the method of Rheinwald and Green [11] and cultured in growth factor and serum-supplemented Dulbecco's modified eagle medium (DMEM)/Ham F12 to confluency. After liquid nitrogen storage, keratinocytes were cultured in keratinocyte serum-free medium (Keratinocyte-SFM, Gibco, Invitrogen Ltd, Paisley, UK).

Immunohistochemistry

Formalin-fixed skin specimens were paraffin-embedded and fifty 4-μm sections were prepared from each biopsy. Using a DAKO AutoStainer device (DakoCytomation N.V., Heverlee, Belgium) every fifth section was stained with a CD117 antibody [17, 48, 59] (c-KIT; dilution 1/600; DAKO, Carpinteria, CA, US) for dermal mast cell quantification and every 5+2 section was stained with an anti-Melan-A antibody (dilution 1/20; Novacastra Laboratories Ltd, Newcastle upon Tyne, UK) for melanocyte quantification. CD117+ mast cells are subsequently referred to as mast cells in the Results section.

Image capturing and image analysis

For quantification of the epidermal melanocyte or dermal mast cell population three non-overlapping, adjacent images were taken from each section. With ten sections being analyzed per biopsy per staining, this resulted in 30 epidermal and 30 dermal images per biopsy. High-resolution (1024x768 dpi) TIFF images were captured using a PixeLINK Megapixel Firewire camera mounted on top of a Zeiss Axiostar Plus light microscope coupled to PixeLINK Capture software (PixeLINK, Ottowa, Canada). These images were imported by our in-house image analysis software environment sRGBToolbox (developed by Yves Vander Haeghen, Department of Dermatology, Ghent University, Ghent, Belgium. Can be downloaded at http://www.uzdermis.ugent.be/yvdh).

Table 1 Patient profiles used in this study

Patient	Age (years)/ sex	Sample	Localization of biopsy	Diagnosis
C1	54 / M	BCC, P	Upper right leg (n + c)	–
C2	52 / F	BCC	Trunk (n + c)	–
C3	28 / F	BCC, P	Right buttock (n + c)	–
C4	28 / F	BCC	Left upper leg (n + c)	–
C5	16 / F	BCC	Right leg (c)	–
C6	22 / M	BCC, BIHC	Trunk (c)	–
C7	25 / F	BCC, BIHC	Left armpit (c)	–
C8	31 / F	BCC	Left upper leg (n + c)	–
C9	27 / F	P	–	–
C10	25 / F	P	–	–
C11	32 / M	P	–	–
C12	50 / F	P	–	-
C13	23 / M	P	–	–
C14	26 / F	P	–	–
C15	45 / F	P	–	–
C16	26 / F	P	–	–
C17	59 / F	P	–	–
C18	27 / F	P	–	–
C19	51 / F	P	–	–
C20	35 / F	P	–	–
C21	23 / F	BIHC	Inside left upper arm (n)	–
C22	21 / M	BIHC	Inside left upper arm (n)	–
C23	41 / F	BIHC	Inside left upper arm (n)	–
C24	63 / F	BIHC	Inside left upper arm (n)	–
C25	22 / F	BIHC	Inside left upper arm (n)	–
C26	33 / M	BIHC	Inside left upper arm (n)	–
C27	37 / M	BIHC	Right buttock (c)	–
C28	20 / F	BIHC	Left lower leg (c)	–
C29	12 / F	BIHC	Left upper leg (c)	–
C30	13 / F	BIHC	Neck (c)	–
C31	23 / M	BIHC	Forehead (c)	–
C32	16 / F	BIHC	Right shoulder (c)	–
C33	22 / F	BIHC	Left wrist (c)	–
NF1	50 / M	BCC	Trunk (n), left upper leg (c)	clinical
NF2	47 / F	BIHC, BCC, P	Trunk (c), right buttock (n)	clinical
NF3	24 / F	BCC	Trunk (c)	Molecular genetic
NF4	46 / M	BIHC, BCC, P	Left upper leg (n + c)	Molecular genetic
NF5	48 / F	BIHC, BCC, P	Trunk (c), buttock (n)	Molecular genetic
NF6	35 / F	BCC	Trunk (n + c)	Molecular genetic
NF7	17 / M	BCC	Trunk (n + c)	Molecular genetic
NF8	35 / F	BCC	Trunk	Molecular genetic
NF9	51 / M	BCC	Trunk (n + c)	Molecular genetic
NF10	40 / M	BIHC, BCC, P	Trunk (c), buttock (n)	NF1 mutation analysis ongoing
NF11	45 / F	BIHC, BCC, P	Trunk (n), right upper arm (c)	Molecular genetic
NF12	10 / M	BIHC, P	Trunk (c)	Molecular genetic
NF13	11 / F	BIHC	Trunk (c)	Clinical
NF14	7 / F	BIHC	Trunk (c)	Clinical
NF15	12 / M	BIHC	Trunk (c)	Clinical
NF16	34 / F	BIHC, BCC	Trunk (c), buttock (n)	Molecular genetic
NF17	45 / F	BCC	Trunk (c + n)	Molecular genetic

Abbreviations: *C* control patient; *NF* Neurofibromatosis type 1 patient; *M* male; *F* femal; *BCC* biopsy for cell culture; *BIHC* skin biopsy for immunohistochemistry; *P* blood plasma; *c* CALM skin, *n* normal skin

Briefly, melanocytes and mast cells were digitally marked directly on the images and the software kept track of the drawn markers. Subsequently, specific regions were highlighted (basal membrane; see Fig. 2c) and/or delineated (dermal area; see Fig. 2g) using the included distance and surface measurement functionality. This functionality automatically calculated the exact distance (basal membrane in mm) or surface (dermal area in mm^2) of the drawn regions based on microscopic magnification and image resolution. Melanocytes per mm basal membrane (via actual distance measurement along the curvature of the basal membrane) and the number of mast cells per mm^2 dermis (by demarcating the dermal surface area) per image per section were counted and enabled us to accurately calculate the mean cell number across the whole specimen.

ELISA

For supernatant collection, 150,000 cells (passage of four fibroblasts and three keratinocytes) were seeded

in a 60-mm petri dish (two dishes of each cell type) with 5 mL of culture medium. After 72 h the cell culture supernatant was collected. An ELISA kit with antibodies raised against an *E. coli*-expressed recombinant human soluble SCF was purchased from R&D Systems (Abingdon, Oxon, UK). It was used according to the manufacturer's guidelines and optical densities were read using a Labsystems Multiscan RC microplate reader set to 450 nm with a wavelength correction at 540 nm. All samples were assayed in duplicate.

Gel electrophoresis and Western blot analysis

Cells were lysed in a mild lysis buffer (10 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.7% NP40, pH 7.4) supplemented with the following protease inhibitors (all from Sigma): 1.72 mM phenylmethylsulfonyl fluoride (PMSF), 21 μM leupeptin and 10 μg/mL aprotinin. Equal amounts of protein were solubilized, denatured by incubating in sample buffer and boiled in the presence of 5% β-mercaptoethanol. Denatured proteins were separated by 6% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted onto PVDF membranes (Amersham Biosciences, Orsay, France). Membranes were blocked for 1 h at room temperature in 5% non-fat dry milk in TBS containing 0.05% Tween-20 (TBS-T), followed by incubation with monoclonal anti-SCF (sc-13126; 1/500) (Santa-Cruz Biotechnology Inc., Santa Cruz, CA, USA) or polyclonal anti-c-KIT (CD117; 1/200) (DAKO, Carpinteria, CA, USA). Following several washing steps with TBS-T, membranes were incubated with anti-mouse (1/3,000) or anti-rabbit (1/4,000) IgG conjugated with horseradish peroxidase (Amersham Biosciences). After the final washing steps with TBS-T, bound antibodies were detected using the enhanced chemiluminescence detection system ECL + Plus (Amersham Biosciences) according to the manufacterer's protocol.

Total RNA isolation, cDNA synthesis and real-time quantitative PCR

RNA was isolated using the RNeasy mini kit (Qiagen, Leusden, The Netherlands) followed by DNase treatment of the RNA (Promega, Leiden, The Netherlands) as described previously [53]. The analysis of the relative gene expression of total cellular and membrane-bound SCF, and c-KIT receptor was performed using real-time (RT) quantitative PCR with the ABI Prism 7000 and the $2^{-\Delta\Delta CT}$ method for relative gene expression [53]. Specific primers for the c-KIT receptor (forward primer 5′- CTGATCCGGGCTTTGTCAA-3′; reverse primer 5′- CATTCATTCTGCTTATTCTC ATTCG-3′) were designed using Primer Express software v2.0 (Applied Biosystems, Lennik, Belgium). The primer sequences for total cellular SCF were previously described [40]. Primers used for detection of membrane-bound SCF were: forward primer 5′-AT CCATTGATGCCTTCAAGGA-3′ and reverse primer 5′-TTCCCTTTCTCAGGACTTAATGTTG-3′. Appropriate internal control genes, used to normalize the PCR for differences in the amount of cDNA added to the reactions, were selected using Genorm [53]. Two-step RT-PCR SYBR green assays were performed using a 25 μl mixture containing 12.5 μl of 2x SYBR green PCR mastermix (Applied Biosystems), 5 μl of cDNA template, 300 nM of forward and reverse primer and 4.5 μl of Rnase-free water (Sigma, Bornem, Belgium). The cycling conditions comprised of 2 min at 50°C, 10 min of polymerase activation at 95°C and 40 cycles at 95°C for 15 sec and 60°C for 60 sec. A dissociation curve from 60° to 95°C was performed after each run to exclude primer-dimer formation.

Statistical analysis

Statistical significance was evaluated by the non-parametric Mann-Whitney *U*-test. The significance level was set at $P < 0.05$.

Results

Melanocyte density is increased in NF1 CALM skin compared with NF1 normal skin, control normal skin and control CALM skin

Only one numerical cell population study has analyzed the in vivo number of epidermal melanocytes in CALM and normal skin of NF1 patients by investigating the epidermal keratinocyte/melanocyte ratio, and found an increase in melanocyte number compared with the normal skin of control patients [9]. However, this study did not include CALM skin of control patients. We obtained biopsies from non-sun-exposed normal skin and CALM skin in NF1 patients and control patients. Two biopsies were taken from sun-exposed CALM skin (neck and face) but because the results were in the same range as the others in the control CALM skin group, they were included in the analysis. By accurate measurement and comparison of the number of melanocytes per mm basal membrane in the four skin groups (control normal skin, control CALM skin, NF1 normal skin, NF1 CALM skin), we found that melanocyte density in NF1 CALM skin was significantly higher compared with the control normal and CALM skin, and even higher compared with the NF1 normal skin. The increase in NF1 normal skin compared with the control normal and CALM skin, however, did not reach statistical significance (Figs. 1a, 2a–d and Table 2).

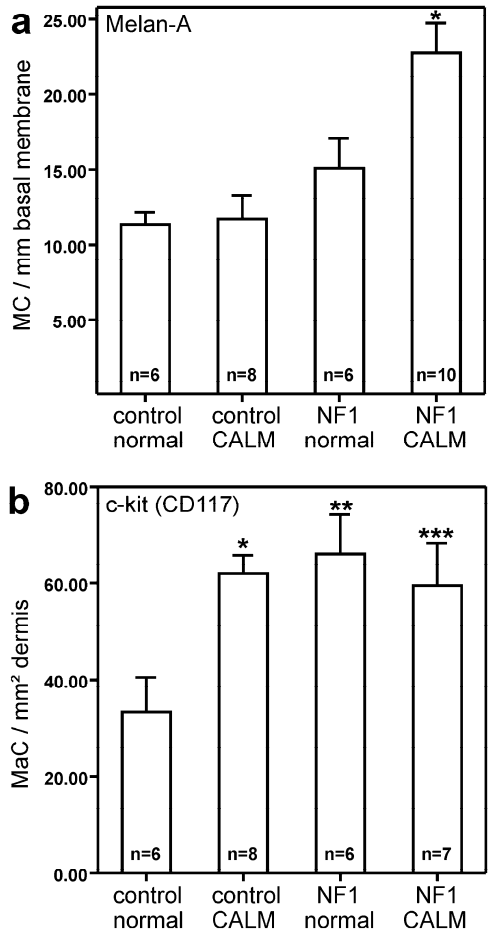

Fig. 1 Quantitative cell population analysis: epidermal melanocytes per mm basal membrane (**a**) and mast cells per mm^2 dermis (**b**) in control normal, control CALM, NF1 normal and NF1 CALM skin. The number of patient biopsies included in each group is highlighted above the *x*-axis (group derivation). (**a**) * statistically significant higher number of melanocytes ($P < 0.05$) in NF1 CALM skin compared with the control normal skin, control CALM skin and NF1 normal skin. (**b**) *,**,*** statistically significant higher number of mast cells ($P < 0.05$) in NF1 normal skin, NF1 CALM skin and control CALM skin compared with the control normal skin

Mast cell densities are significantly higher in NF1 CALM skin, NF1 normal skin and control CALM skin compared with control normal skin

An earlier study has found an increase in the in vivo number of mast cells in CALM and normal skin of NF1 patients compared with the normal skin of control patients [40]. However, mast cell density analysis did not include CALM skin of control patients. We accurately determined the number of c-KIT + mast cells using CD117 staining, as these cells are potentially receptive to fibroblast-derived KIT ligand (SCF). Surprisingly, the number of mast cells did not show the same difference between control skin and NF1 skin as we found in melanocytes. Mast cell numbers in control CALM skin, NF1 normal and CALM skin were significantly higher than control normal skin. However, the three increased groups did not show any statistically significant difference among themselves (Figs. 1b, 2e, f and Table 2). In other words the difference between NF1 CALM and control CALM was in the increased number of melanocytes, and not in mast cell number.

Soluble SCF levels are increased in NF1 fibroblast supernatant (normal and CALM skin) compared with control fibroblast supernatant (normal and CALM skin)

As increased secretion of dermal fibroblast-derived SCF (soluble SCF) may be associated with the accentuated epidermal melanization in CALM skin of NF1 patients [40], we thought it would be interesting to see if this was also the case in the control CALM skin group. SCF secretion was also analyzed in keratinocyte supernatant of the four skin groups to confirm and expand the previous study. SCF secretion was assayed via ELISA. The amount of soluble SCF detected in keratinocyte supernatant from control normal skin and CALM skin did not show a significant difference to the amount in NF1 normal and CALM skin nor to the amount detected in serum-free keratinocyte medium (negative control) (Fig. 3a). However, the amount of soluble SCF detected in NF1 fibroblast supernatant was significantly higher compared with the fibroblast supernatant in control normal skin and control CALM skin (Fig. 3b).

In neither control nor NF1 patients there was a significant difference between normal skin and CALM skin. These results confirm that soluble SCF might indeed be important not only in NF1 CALM skin but also in NF1 normal skin, and that soluble SCF is probably not the cause of mast cell proliferation in control CALM skin. In addition, we used ELISA to determine the circulating SCF levels in plasma of 13 control individuals and 6 NF1 patients. We did not find a significant difference between the two groups (data not shown).

Total cellular (intracellular and membrane-bound) SCF, and c-KIT receptor mRNA expression do not significantly differ between control and NF1 skin fibroblasts and keratinocytes

We first determined total cellular SCF mRNA expression in fibroblasts and keratinocytes obtained by using RT quantitative PCR using primers located in exon 1. Expression levels did not significantly differ between the four groups (data not shown). As increase in secretion of soluble SCF in NF1 fibroblasts could be the result of lower expression of the SCF splice variant lacking exon 6 (which codes for the membrane-bound form or mSCF) and therefore favoring full-length mRNA, we analyzed the expression of membrane-bound SCF mRNA using primers located on the exon 5–exon 7 boundary.

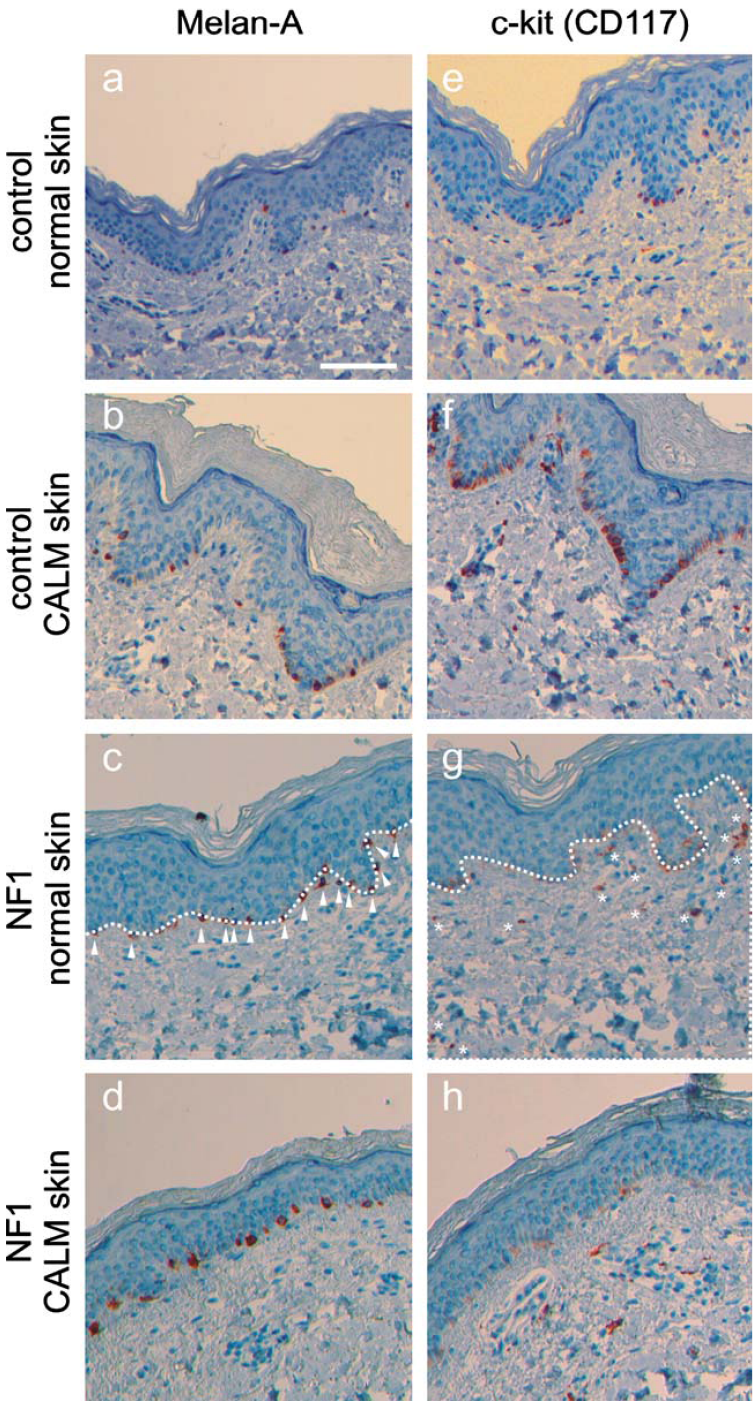

Fig. 2 Representative immunohistological staining of epidermal melanocytes and dermal mast cells. This figure visually demonstrates melanocyte and mast cell density and illustrates the counting method. A representative image of every group is shown: Melan-A stain of control normal (**a**), control CALM (**b**), NF1 normal (**c**) and NF1 CALM skin melanocytes (**d**) showing melanocytes in the basal layer of the epidermis. (**c**) Melanocytes (*arrows*) along the curvature of the basal membrane (*dotted line*) were identified, counted and measured (cells per mm basal membrane) using the image analysis software sRGBToolbox (see Material and methods). c-KIT (CD117) staining in adjacent sections showing c-KIT + mast cells in the upper dermis (and c-KIT + melanocytes in the epidermis) of (**e**) control normal, (**f**) control CALM, (**g**) NF1 normal and (**h**) NF1 CALM skin. Mast cells [*asterisks* – see (**g**)] are identified, counted and measured (cells per mm^2) within the dermal surface area (*dotted line*; indicating boundary of cell count and skin depth). Scale bar = 100 μM

Table 2 Epidermal melanocyte and dermal mast cell quantification (mean ± SEM)

	Control normal skin	Control CALM skin	NF1 normal skin	NF1 CALM skin
Melanocytes/mm basal membrane	11.28 (±0.81)	11.67 (±1.59)	15.08 (±1.99)	22.82 (±1.91)
Mast cells/mm^2 dermis	33.38 (±7.34)	62.04 (±3.81)	66.13 (±8.11)	59.51 (±8.90)

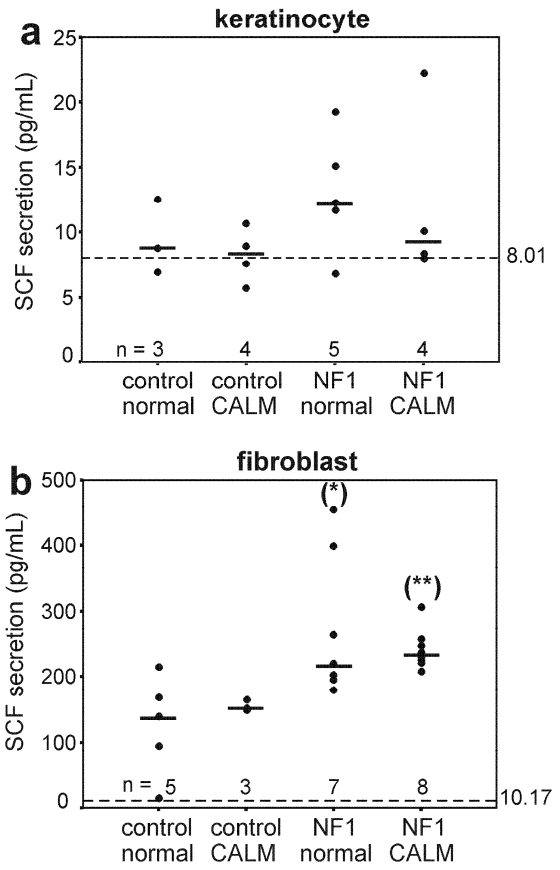

Fig. 3 Secretion of soluble SCF in (**a**) keratinocyte culture supernatant and (**b**) fibroblast culture supernatant of control normal and CALM skin and NF1 normal and CALM skin. SCF secretion was assayed using ELISA and is represented *as dot plots* (pg/mL). The *short horizontal line* in each group represents the median. (**a**) No significant differences were detected between the four groups of keratinocyte supernatant. The *dashed horizontal line* represents the SCF level in keratinocyte serum-free medium used as a negative control. (**b**) *Dot plot* showing SCF secretion in fibroblast culture supernatant from control normal and CALM skin and NF1 normal and CALM skin. *, ** The SCF levels in fibroblast culture supernatant from NF1 skin were significantly higher compared with the control skin. The *dashed horizontal line* represents fibroblast medium analyzed as negative control

However, we did not find any differences between the four groups (data not shown). The c-KIT mRNA expression levels in melanocytes from the four groups were not significantly different (data not shown).

Total cellular (intracellular and membrane-bound) SCF protein expression does not differ between control and NF1 skin fibroblasts and keratinocytes. Control CALM melanocytes display a higher protein expression of the c-KIT receptor

Using western blot analysis we did not find a difference in total cellular SCF protein expression in keratinocytes obtained from control and NF1 skin (Fig. 4a). SCF expression in fibroblasts was again the same in the control group compared with the NF1 group (Fig. 4a).

The c-KIT receptor protein was detected in melanocytes obtained from control normal and CALM skin and NF1 normal and CALM skin. A higher expression was observed in control CALM melanocytes compared with control normal skin melanocytes and NF1 normal and CALM skin melanocytes. The differences between the control CALM group and the control normal and NF1 normal skin group presented borderline statistical significance ($P = 0.05$) (Fig. 4b).

Discussion

Melanocyte density in normal skin varies in different regions of the body with the highest densities on the face and male genitals (about 2000/mm^2) and the lowest on the trunk (about 800/mm^2) [51]. The number of melanocytes also decreases with age [41].

Differences in racial pigmentation are not caused by differences in melanocyte number but by a different number and distribution of melanosomes. The pathophysiology of CALM is still largely unknown. Several researchers have tried to characterize CALM histologically resulting in conflicting results. Some studies showed an increased melanocyte density in NF1 CALM and in NF1 normal skin [4, 9, 27], while others showed this increase solely in NF1 CALM but not in their normal skin [23, 52]. Ishida and Jimbow [19] found the melanocyte density in NF1 CALM to depend on their pigmentation with a normal number of melanocytes compared with the normal surrounding skin in light brown to brown macules and a significant increase in the number of dark brown CALM. In a more recent study where laser treatment of CALM from both NF1 patients and control individuals was studied two histologic subtypes were found with either normal numbers of melanocytes or increased numbers. The two types were found in both NF1 and control individuals [13]. On the other hand, isolated CALM in the general population were found to have fewer DOPA-positive melanocytes per mm^2 than the surrounding skin [23]. A Japanese study compared pigmented macules in naevus spilus with normal surrounding skin and found no differences in melanocyte counts in six patients and a very slight elevation in the other three patients [52]. CALM in ataxia-telangiectasia were found to have a normal number of melanocytes but an increase in synthesis of melanosomes and a different melanosomal distribution in the epidermal keratinocytes [43]. Comparison of absolute numbers of melanocytes between different studies is difficult due to the use of different dimensions (one- or two-dimensional values).

It is clear that the discussion concerning melanocyte number in CALM remains. To our knowledge our study is the first large-scale study on melanocyte and mast cell numbers in normal skin and CALM skin of control and

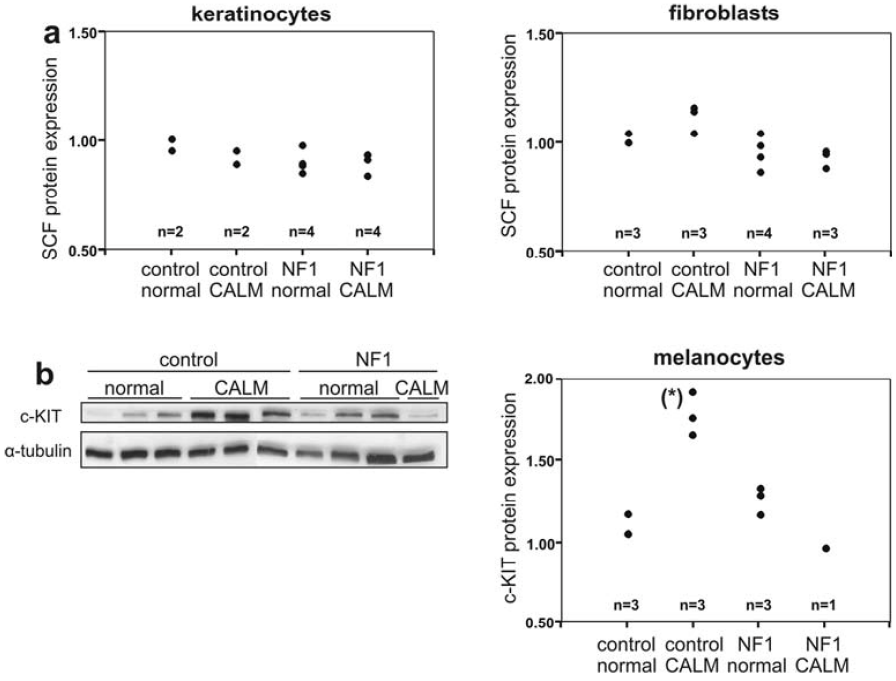

Fig. 4 Total cellular SCF protein expression in epidermal keratinocytes and dermal fibroblasts, and c-KIT receptor protein expression in epidermal melanocytes. (**a**) *Dot plot* representing total cellular (intracellular and membrane-bound) SCF protein expression in keratinocytes and fibroblasts obtained from control normal, control CALM, NF1 normal and NF1 CALM skin. The number of included samples is indicated by *n* above the *x*-axis. (**b**) c-KIT receptor expression in melanocytes obtained from control and NF1 normal and CALM skin is represented in a *dot plot*. Intensity of the band for c-KIT was corrected to that for alpha-tubulin. * Borderline statistically significant increase ($P = 0.05$) in c-KITreceptor protein expression in control CALM melanocytes compared with the control and NF1 normal skin melanocytes. The number of patient biopsies included in each group is highlighted above the *x*-axis (group derivation)

NF1 patients. For the first time more than 180 high-power fields were carefully examined in each of the groups using a more standardized and software-assisted distance and surface measurement tool.

We biopsied only the typical light to medium brown (coffee with milk) CALM and found an increase in melanocyte density in NF1 skin with a statistically significant increase in NF1 CALM skin compared with the control normal and CALM skin, and also compared with the NF1 normal skin. The higher number of melanocytes in NF1 normal (non-lesional) skin, although not statistically significant, might help to explain the grey-brown complexion that we often clinically observe in NF1 patients.

Little is known about the normal number of mast cells in control skin, let alone in CALM skin. Mast cell numbers in normal skin of 60 healthy Indian volunteers showed higher numbers in a younger age group (20–25 years old) compared with the older group for both sexes and in facial skin compared with arm skin [5, 56]. Mast cells have been known to be increased in uninvolved and lesional skin of psoriasis patients and in lesional skin in lichen planus [56], atopic dermatitis [8], bullous pemphigoid [38] and pemphigus vulgaris [31] but not in chronic idiopathic urticaria [50]. In NF1 however, no correlation between mast cell density and age of the NF1 patient was found [39]. Moreover, the increase in mast cells in neurofibromas was statistically significant compared with the NF1 normal skin [24, 39]. Recently the first (and to our knowledge the only) description of mast cell numbers in NF1 CALM revealed a marked increase in mast cells in NF1 CALM and to a lesser degree also in NF1 normal skin compared with the normal skin of control individuals [40]. We also found a statistically significant increase in mast cells in NF1 skin. Additionally an increase in mast cell number in control CALM skin was observed. In our study we analyzed the mast cell population, as they are potentially KIT-ligand (SCF)-receptive. To our knowledge this is the first time that solitary CALM of control individuals are included in the analysis and therefore the first time that histological differences in melanocyte and mast cell number between isolated and NF1-associated CALM are described. Absolute mast cell numbers per mm^2 dermis, detected in control normal skin and NF1 normal and CALM skin, were comparable with those reported in previous studies [1, 8, 20, 24, 38–40].

One study indicated that this increased number of mast cells (and melanocytes) in NF1 CALM skin could be attributed to the secretion of growth factors, such as SCF and HGF by dermal fibroblasts [40]. We decided to further investigate the role of SCF and include isolated control CALM (which do not show the increased number of melanocytes) in the analysis.

SCF has been shown to affect melanocyte and mast cell growth, survival, secretion and cohesion as well as migration into tissues [10]. SCF has also been shown to be important in UVB-induced pigmentation [18], in lentigo senilis [15] and in dermatofibroma [47]. A recent study [57, 58] in Nf1-mutant mice showed that $Nf1^{-/-}$ melanocyte precursors did not require SCF for their survival and that they migrated normally on the lateral crest pathway. In addition Nf1-mutant melanocytes were more sensitive to SCF than wild-type melanocytes. Apart from its influence on melanocytes, SCF also causes accumulation of mast cells in the dermis. Several publications cover the possible role of SCF in mastocytosis. Kunisada et al. [30] showed that in mice keratinocyte expression of transgenic SCF results in accumulation of mast cells within the dermis and epidermal melanocyte maintenance and melanogenesis, a phenotype resembling human mastocytosis. Two case reports described the association between mastocytosis and NF1: one patient had a combination of NF1 and systemic mastocytosis [35] and a second one showed lesions of urticaria pigmentosa with CALM and cutaneous and plexiform neurofibromas [36].

As could be expected we did not detect soluble SCF in keratinocyte culture supernatant because keratinocytes predominantly express the strictly membrane-bound form of SCF. We did find an increased secretion of SCF by NF1 normal and CALM fibroblasts, which might explain the increased melanocyte and mast cell density in NF1 skin. Surprisingly, our data showed that SCF mRNA and cell-associated protein expression did not differ between NF1 and control fibroblasts, and that increased SCF secretion could not be explained by lower expression of the SCF splice variant lacking the cleavage site for release of membrane-bound SCF. Maybe this could indicate that increased secretion of SCF by fibroblasts might be due to a higher enzymatic cleavage rate of membrane-bound SCF. The proteases producing this cleavage are unknown. A potential candidate is a chymotrypsin-like protease, called chymase, which is known to predominate in connective tissues, including the dermis [32]. The recent study by Okazaki et al. [40] also reported the higher SCF secretion by NF1 CALM fibroblasts compared with the healthy control normal skin, with especially high values in two out of five patients. They however did not find a statistically significant increase in SCF secretion by NF1 normal skin fibroblasts, nor the high levels of mast cells in NF1 normal skin. In their, as in our study, normal skin samples were not overlying or adjacent to neurofibromas which might influence the results. This could point to a large interpatient variability in the influence of SCF in NF1 normal skin. In addition, in their study they also found an increased SCF mRNA expression in two patients using RT quantitative PCR and in two out of three patients using semi-quantitative RT-PCR. They did not however confirm these results on a protein level.

Increased levels of circulating growth factors such as SCF and midkine in NF1 patient serum have been suggested to stimulate growth of neurofibroma-derived cells [34]. We also detected circulating SCF levels in our NF1 patient population and in a number of controls. It has already been shown that there is no correlation between SCF levels and gender, age or tumor burden in NF1 patients [34]. The circulating plasma SCF in controls that we detected was in the same range as in the previously published results for normal individuals [29]. However, even though NF1 plasma SCF levels were higher compared with the controls the difference was not statistically significant. We cannot explain this discrepancy except that our patient population might be too small to detect a significant difference.

The c-KIT receptor has been shown to be expressed in melanocytes, mast cells and epithelial cells in the hair follicle [44]. We know of no previous reports on c-KIT receptor expression in CALM. We found a higher protein (but not mRNA) expression in control CALM melanocytes, which might make these melanocytes more susceptible to the effects of SCF and consequently activate them.

In conclusion and as summarized in Table 3, in NF1 patients we did not detect any differences between normal and CALM skin in mast cell numbers, SCF

Table 3 Overview of results

	Control normal skin	Control CALM skin	NF1 normal skin	NF1 CALM skin
Melanocyte number/mm basal membrane	=	=	↑	↑↑
Mast cell number/mm^2 dermis	=	↑↑	↑↑	↑↑
sSCF secretion in KC supernatant (pg/ml)	=	=	=	=
sSCF secretion in FB supernatant (pg/ml)	=	=	↑↑	↑↑
SCF mRNA expression (KC/FB)	=	=	=	=
SCF protein expression (KC/FB)	=	=	=	=
c-KIT mRNA expression (MC)	=	=	=	=
c-KIT protein expression (MC)	=	↑↑	=	=

Abbreviations: = normal; ↑ increase; ↑↑ statistically significant increase; *KC* keratinocyte; *FB* fibroblast; *MC* melanocyte

secretion and SCF mRNA and protein expression in fibroblasts and keratinocytes. The only distinction was in the significantly higher melanocyte density in NF1 CALM skin. We already know from previous studies [26] that these NF1 CALM melanocytes display a higher melanin content and melanogenesis. The reason for both increased melanocyte number and melanogenesis however remains unclear. One study suggested that NF1 haploinsufficiency influences number, length and angle of melanocyte dendrites and thus affects migration of melanocyte precursors, resulting in CALM [28]. We do not believe that secretion of soluble SCF by dermal fibroblasts can be accounted for CALM formation since we found no differences in SCF levels between normal and CALM skin.

In control individuals the difference between normal and CALM skin lay solely in their mast cell numbers. To our knowledge melanin content and melanogenesis have not yet been studied in control CALM melanocytes (except for the stronger DOPA-reaction detected in pigmented macules of nevus spilus [52]) and we are currently looking into this. Again we do not believe that SCF is the cause for the hyperpigmentation of control CALM (or for the mast cell proliferation) although we cannot exclude the influence of an increased expression of the c-KIT receptor. We could however not confirm this increased expression of c-KIT on the mRNA level, which might suggest that the c-KIT receptor levels are regulated by post-translational or degradational modifications.

The objective of this study was to see whether the mechanism of hyperpigmentation of control and NF1 CALM could be considered as identical. We can confirm that CALM in control and NF1 patients, although macroscopically identical, differ in melanocyte density and the amount of SCF secreted by their fibroblasts. However, no differences in SCF mRNA and protein expression could be detected. We suggest that, in NF1 fibroblasts, a higher enzymatic cleavage of the anchored SCF precursor, might release larger quantities of the soluble growth factor without higher expression levels.

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Goal of the thesis

In Neurofibromatosis type 1 patients the NF1 gene is mutated. Several cardinal features of NF1 comprise cutaneous and non-cutaneous pigmentary defects (see **chapter 1 – article I**) which could argue for a specific role of neurofibromin in regulation of melanocyte function / homeostasis. The major goal of this study was to gain more insight into the functional role of the NF1 gene and its corresponding gene product in primary human epidermal melanocytes. To achieve this goal the following research objectives were proposed:

- **A (high throughput) gene / mRNA level approach:**
  Addressing the effects of NF1 heterozygosity on total gene expression of primary human epidermal melanocytes: towards functional analysis of the NF1 gene.

  By using a high-throughput transcriptome expression analysis via cDNA microarray technology, we tried to establish information on the role of the NF1 gene by looking into a human NF1 heterozygous ($NF1^{+/-}$) melanocytic cell system. Moreover, this is the first analysis of its kind in primary human epidermal melanocytes with haploinsufficient expression of the NF1 gene locus. The specific experimental setup enabled us to look for differentially modulated genes that were expressed in a genotype ($NF1^{+/+}$ vs. $NF1^{+/-}$) and / or lesional type (normally pigmented versus hyperpigmented CALM skin) dependent fashion. This objective was a first (indirect) attempt to learn more about the role of the wild type NF1 gene and its product neurofibromin in the regulation of melanocyte biology. The results of this study are described and discussed in **chapter 2**.

- **A RNA interference (RNAi)-mediated approach:**
  Analyzing the effects of endogenous neurofibromin suppression on proliferation and cell cycle distribution of primary human epidermal melanocytes.

  Via RNA intereference (RNAi)-mediated knock-down of endogenous NF1 gene (product) levels we analysed the effects on melanocyte proliferation and cell cycle distribution. **Chapter 3** reports the results of this study.

- **A (subcellular) protein level approach:**
  Investigation of the intracellular localization and association of neurofibromin with a new interaction partner in primary human epidermal melanocytes.

  Via yeast two hybrid, co-immunoprecipitation and subcellular / ultrastructural analysis a new neurofibromin interaction partner was identified in epidermal melanocytes, namely amyloid precursor protein (APP). Both constituents were shown to colocalize with melanosomes. This subcellular localization / association was abrogated in an NF1 heterozygous background. The results of this subcellular localization study are described in **chapter 4**.

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Unraveling the effects of NF1 heterozygosity in human epidermal melanocytes: towards functional analysis of the NF1 gene

Article III

Gene expression profiling of cultured human NF1 heterozygous ($NF1^{+/-}$) melanocytes reveals downregulation of a transcriptional cis-regulatory network mediating activation of the melanocyte-specific dopachrome tautomerase (DCT) gene.

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Gene expression profiling of cultured human *NF1* heterozygous (*NF1*$^{+/-}$) melanocytes reveals downregulation of a transcriptional cis-regulatory network mediating activation of the melanocyte-specific dopachrome tautomerase (*DCT*) gene

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Summary

One of the major primary features of the neurocutaneous genetic disorder Neurofibromatosis type 1 are the hyperpigmentary café-au-lait macules where disregulation of melanocyte biology is supposed to play a key etiopathogenic role. To gain better insight into the possible role of the tumor suppressor gene *NF1*, a transcriptomic microarray analysis was performed on human *NF1* heterozygous (*NF1*$^{+/-}$) melanocytes of a Neurofibromatosis type 1 patient and *NF1* wild type (*NF1*$^{+/+}$) melanocytes of a healthy control patient, both cultured from normally pigmented skin and hyperpigmented lesional café-au-lait skin. From the magnitude of gene effects, we found that gene expression was affected most strongly by genotype and less so by lesional type. A total of 137 genes had a significant twofold or more up- (72) or downregulated (65) expression in *NF1*$^{+/-}$ melanocytes compared with *NF1*$^{+/+}$ melanocytes. Melanocytes cultured from hyperpigmented café-au-lait skin showed 37 upregulated genes whereas only 14 were downregulated compared with normal skin melanocytes. In addition, significant genotype × lesional type interactions were observed for 465 genes. Differentially expressed genes were mainly involved in regulating cell proliferation and cell adhesion. A high number of transcription factor genes, among which a specific subset important in melanocyte lineage development, were downregulated in the cis-regulatory network governing the activation of the melanocyte-specific dopachrome tautomerase (*DCT*) gene. Although the results presented have been obtained with a restricted number of patients (one NF1 patient and one control) and using cDNA microarrays that may limit their interpretation, the data nevertheless addresses for the first time the effect of a heterozygous *NF1* gene on the expression of the human melanocyte transcriptome and has generated several interesting candidate genes helpful in elucidating the etiopathology of café-au-lait macules in NF1 patients.

Key words: NF1/heterozygosity/café-au-lait macule/melanocyte/DCT/microarray

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Introduction

The melanocyte is a distinct cell type that develops from a non-pigmented melanocyte precursor in the neural crest, called the melanoblast (Le Douarin and Ziller, 1993). Melanocytes are found in the basal layer of skin epidermis, in hair follicles, in the inner ear and in the eye (Boissy, 1988). In epidermal skin their specialized function comprises melanin biosynthesis and transfer to surrounding keratinocytes through a combined process of melanogenesis and dendritogenesis, therefore providing an effective protection against UV-induced DNA damage (Eller et al., 1996; Iyengar, 1998). These processes involve melanocyte-specific differentiation pathways that result in a unidirectional, sequential maturation of lysosome-related organelles, called melanosomes, where enzyme-mediated biosynthesis and deposition of melanin pigment occurs. The melanosomes are actively transported by motor proteins along a microtubular network in specific cellular protrusions, called the dendrites, towards a subcortical actin network

in the dendrite tips where they accumulate and are transferred by an unknown molecular mechanism to surrounding keratinocytes (Lambert et al., 1998; Vancoillie et al., 2000; Westbroek et al., 2003). Three major melanosome accessory enzymes of the tyrosinase gene family are involved in melanin biosynthesis. Tyrosinase (TYR) is the rate-limiting enzyme mediating conversion of the amino acid tyrosine to 3,4-dihydroxyphenylalanine (DOPA) and of DOPA to dopaquinone (Tripathi et al., 1992). After spontaneous conversion of dopaquinone to dopachrome, a second enzyme called tyrosinase-related protein-2 or dopachrome tautomerase (TRP-2/DCT) catalyzes conversion of dopachrome to 5,6-dihydroxyindole-2-carboxylic acid (DHICA) (Tsukamoto et al., 1992). Subsequently, DHICA is converted to indole-quinone-carboxylic acid by tyrosinase-related protein-1 (TRP-1) (Kobayashi et al., 1994). The enzymes TRP-1 and TPR-2/DCT are involved in the biosynthesis of eumelanin, the black-brown pigment with the highest photoprotective capabilities (Kobayashi et al., 1995).

A complex cascade of extracellular and intracellular signals controls melanocyte development, growth and function (Sieber-Blum, 1998; Wehrle-Haller and Weston, 1997). Among the extracellular cues are several para- and autocrine regulatory cytokine networks [stem cell factor (SCF), endothelin-1 (ET-1), basic fibroblast growth factor (bFGF)] that mediate cell–cell signaling between melanocytes and surrounding epidermal keratinocytes and dermal fibroblasts (Imokawa, 2004). The subsequent downstream effect of the cytokine-induced or cell–cell contact mediated signaling is the activation of specific target genes, of which expression is regulated by transcription factors (inducers–repressors). Several transcription factors are involved in normal melanocyte development, such as PAX3 (Galibert et al., 1999), SOX10 (Mollaaghababa and Pavan, 2003) and MITF (Widlund and Fisher, 2003). It appears that the basic helix–loop–helix leucine zipper (bHLH-LZ) microphthalmia-associated transcripion factor (MITF), and in particular the melanocyte-specific isoform MITF-M, can be centered in the complex network of transcription factors that regulate normal melanocyte development (Vance and Goding, 2004). Together with the crucial role of the type III receptor tyrosine kinase KIT, and its ligand SCF, in initiating a signal transduction cascade which has been found to trigger phosphorylation of MITF, these extracellular and intracellular key players seem to fulfill a crucial role in development of the melanocyte lineage (Hou et al., 2000; Opdecamp et al., 1997; Wehrle-Haller, 2003).

Hypo- and hyperpigmentary disorders are etiopathogenically characterized by disregulation of melanocyte development, survival, proliferation and/or differentiation. This leads to constitutive activation or inactivation of lesional melanocytes and can be caused by changing microenvironmental cues because of distorted cytokine and chemokine signaling and/or specific (abnormal) interactions with surrounding epidermal keratinocytes, dermal fibroblasts and mast cells (Imokawa, 2004). Otherwise it is known that several pigmentary disorders are caused by mutations of melanocyte-specific genes or key genes involved in melanocyte proliferation and differentiation: oculocutaneous albinism type 1 (*TYR*) (Toyofuku et al., 2001), Waardenburg syndrome type 2A (*MITF*) (Tachibana, 1997), Griscelli-Prunieras syndrome type II (*RAB27A*) (Westbroek et al., 2004).

As a neurocutaneous autosomal dominant disease, Neurofibromatosis type 1 (NF1) is characterized by the presence of benign, hyperpigmentary cutaneous defects [café-au-lait macules (CALMs), inguinal freckling] as well as non-cutaneous defects (iris Lisch nodules) (De Schepper et al., 2005). The responsible *NF1* gene was identified and sequenced in 1990 (Cawthon et al., 1990), is cytogenetically mapped to chromosome 17q11.2, spans approximately 335 kb of genomic DNA (Li et al., 1995) and produces a transcript of 11–13 kb long (Buchberg et al., 1990) with an open reading frame of 8454 nucleotides. The full-length gene product consists of 2818 amino acids and is called neurofibromin (Marchuk et al., 1991). In general NF1 patients harbor one mutated *NF1* allele and this heterozygous genetic constitution results in haplo-insufficient expression of neurofibromin (Griesser et al., 1995). Functionally, neurofibromin is known to downregulate mitogenic activity of the oncogene Ras. A central domain of neurofibromin shows sequence similarity to the family of GTPase-activating proteins (GAPs) and is able to convert Ras from its active, GTP-bound form to its inactive, GDP-bound form by stimulating the slow intrinsic GTPase activity of Ras (Buchberg et al., 1990). This central domain of neurofibromin is called the GAP-related domain (GRD). The *NF1* gene is a tumor suppressor gene as mutations in both *NF1* alleles (loss of heterozygosity – LOH) have been found in malignant tumors associated with NF1 (Arun and Gutmann, 2004). Contrary to NF1 malignant tumors and certain benign tumors, like neurofibromas, no LOH was found in CALM melanocytes of NF1 patients (Eisenbarth et al., 1997).

Genome-wide transcriptomic microarray analysis is an ideal tool to study gene expression patterns and the underlying etiopathology of pigmentary skin disorders, and in particular melanocytic lesions where disregulation of melanocyte biology occurs (Loftus and Pavan, 2000). To examine the nature of genes differentially expressed between normal and pathologic states, it has been suggested to compare skin biopsies obtained from lesional tissue to non-lesional tissue. The use of in vitro primary cell cultures or representative cell lines could be of limited benefit in understanding a specific cutaneous pathophysiology because cultured cells undergo gene expression alterations because of removal from their native microenvironment (Seykora et al., 2003). However, to unravel the effects of a systemic gene mutation on the transcriptomic expression of a specific cell type,

the use of a 'tissue specimen approach' might be of limited use because of the influence of 'contaminating' surrounding cell populations. The use of laser capture microdissection (LCM) on histological cryosections could ease the in vivo isolation of specific cell clusters, e.g. hyperproliferating melanocytes in nevi and melanoma (Seykora et al., 2003) or neuroblastoma cells in neuroblastoma tissue specimens (De Preter et al., 2003). Only, the melanocyte population of normal human epidermal skin and certain cutaneous pigmentary lesions show a spatially periodic, non-clustered distribution of melanocytes. The use of single-cell LCM might have been an option to purify a cell population but would be too laborious and would still generate the increased risk of contaminating surrounding keratinocytes and fibroblasts in further downstream applications. Primary human melanocyte cultures from neurocutaneous genetic disorders like NF1 can be an excellent way of investigating the effect of gene mutations in a pure cell population if in vivo isolation of single cells poses practical problems.

It is still unclear how a mutated *NF1* allele and haploinsufficient expression of neurofibromin affect the etiopathogenesis of specific benign manifestations in NF1 patients. In order to study the role of the *NF1* gene, we performed cDNA microarray analysis to compare the transcript levels of ~13 850 unique human genes of *NF1* heterozygous ($NF1^{+/-}$) melanocytes and *NF1* wild type ($NF1^{+/+}$) melanocytes, cultured from normally pigmented skin and hyperpigmented CALM skin. This study enabled us to identify functionally relevant classes of genes and monitor the effects of genotype and lesional type variation on the expression of the melanocytic transcriptome. In this way, we were able to dissect, at least in vitro, the role of the *NF1* gene in melanocytes. These cells are considered to be the primary (neoplastic) cells in the etiopathogenesis of CALMs in NF1, analogous to another neural crest derivative, the Schwann cell, in neurofibromas (Lukacs et al., 1997). Detailed analysis identified several distinct classes of modulated genes which were mainly involved in controlling cell proliferation and cell adhesion. A subset of transcription factor encoding genes – involved in a cis-regulatory network mediating the activation of melanocytic genes like *TRP-1* and *DCT* – was downregulated in $NF1^{+/-}$ (CALM) melanocytes. The analysis and comparison of gene expression differences between melanocytes with normal and mutated gene states, can redirect investigative pathways and can further help in our understanding of the etiopathological processes in NF1.

Results and discussion

Experimental setup and data analysis

The four combinations of genotype ($NF1^{+/-}$ and $NF1^{+/+}$) and skin lesional type (normal skin and hyperpigmented CALM skin) were each represented by four hybridizations on eight microarrays, according to a loop design (Figure 1). In total 20 988 expressed sequence tags (ESTs) were spotted, which represented ~13 850 unique genes. We excluded 11 643 clones from the analysis because no consistent positive signal was detected across the 16 hybridization samples. Of the 9345 clones having an overall positive signal, 8071 were from known genes (representing 6781 unique genes).

We used a mixed-model approach (see Materials and methods) and applied the REML procedure to produce estimates of the gene-specific treatment effects (genotype, skin lesional type and genotype × skin lesional type interaction) along with the corresponding P-values. Subsequently, we applied the approach as described by Storey and Tibshirani (2003) to calculate *q*-values for each expression difference. Then, by calling treatment or expression differences with *q*-values less than or equal to some threshold *i* significant in a genome-wide test of significance resulted in a FDR ≤ *i* among the significant differences. Visualizations of the significance and the magnitude of the genotype and skin lesional

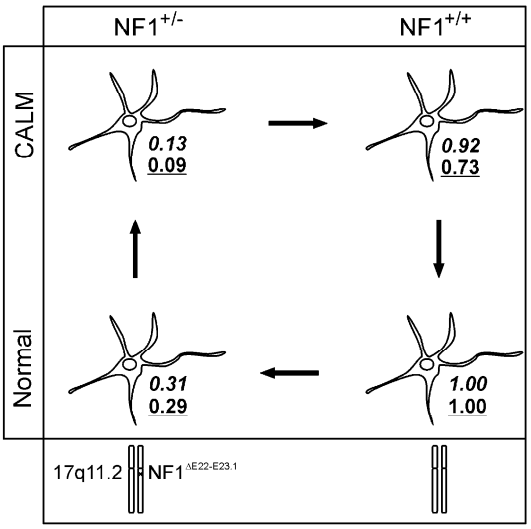

Figure 1. Experimental design: this schematic overview outlines the experimental setup of the microarray experiment. Four major melanocyte groups can be distinguished, based on genotype ($NF1^{+/-}$ vs. $NF1^{+/+}$) and skin lesional type (normally pigmented skin vs. hyperpigmented CALM skin). The NF1 patient has a deletion of exon 22 and a partial, not yet fully determined deletion of exon 23 (ΔE22–E23.1). Individual cDNA microarrays are represented as arrows in the diagram. The sample at the tail of each arrow is labeled with Cy3 and the sample at the head of the arrow is labeled with Cy5. In this arrangement, each sample is labeled equally often with Cy3 and with Cy5 to ensure that expression levels will be free from biases because of dye effects (Churchill and Oliver, 2001). The experiment involved two hybridizations for two mRNA samples, in which the biologically replicated samples are hybridized in the second hybridization. For each melanocyte group the *NF1* gene mRNA expression results obtained with q-RT-PCR (underlined) and microarray (italics) are shown.

type effects were provided by volcano plots (Figure 2), where each point represents one of the 9345 genes having an overall positive signal. These volcano plots contrast significance on the $-\log_{10}$ (q) scale against the expression difference on the $\log_2$ scale. In these volcano plots substantial differences are illustrated as opposed to cut-offs strictly based on the fold change: the two vertical reference lines indicate the (arbitrarily chosen) twofold cut-off for either down- or upregulation of gene expression, whereas the horizontal line represent the preset false discovery acceptance level of 0.0001 ($-\log_{10} = 4$). By setting these two cut-offs, the volcano plot is divided in six sections. Although it is clear from the volcano plot that genes can achieve statistical significance despite their modest fold changes, we decided to focus on those genes where the two methods are in agreement: statistically based significance and significance as determined by the twofold change criterion. According to this selection criteria and after exclusion of redundant transcripts, 137 genes were differentially expressed between the two genotypes: 72 were induced and 65 were repressed in *NF1*$^{+/-}$ compared with *NF1*$^{+/+}$ melanocytes. The full list of 'genotype' differentially expressed genes are viewable in Appendix S1 (see Supplementary material). In melanocytes cultured from hyperpigmented CALM skin, 37 were upregulated whereas only 14 were downregulated compared with the normal skin. The full list of 'lesional type' differentially expressed genes are viewable in Appendix S2 (see Supplementary material). In addition, significant genotype × skin lesional type interactions were observed for 465 genes. Of these genes, 183 were upregulated and 282 were downregulated in the *NF1*$^{+/-}$ genotypic background when lesional CALM melanocytes were compared with normal skin melanocytes (see Appendix S3 (see Supplementary material)). A percentual distribution of differentially expressed genes is depicted in Table 1.

Modulated genes display widespread and preferential chromosomal distribution

Differentially modulated known genes covered all human chromosomes with a strong preference for chromosomes 17, 19 and 22 (Figure 3). These cytogenetic locations could harbor interesting differentially modulated genes. Koga et al. (2002) even described that chromosome imbalances and losses frequently involve chromosomes 17, 19, and 22q in NF1-related neurofibromas and sporadic neurofibromas, and that mainly the cytoband subregions 17p11.2-p13, 17q24-q25 and 19p13.2 were involved in NF1-associated neurofibromas. Several interesting modulated genes in our dataset are cytogenetically mapped to region 17p11.2-p13: peripheral myelin protein 22 (*PMP22*); serine (or cysteine) proteinase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium derived factor), member 1 (*SERPINF1*); active BCR related gene (*ABR*). Region 17q24-q25

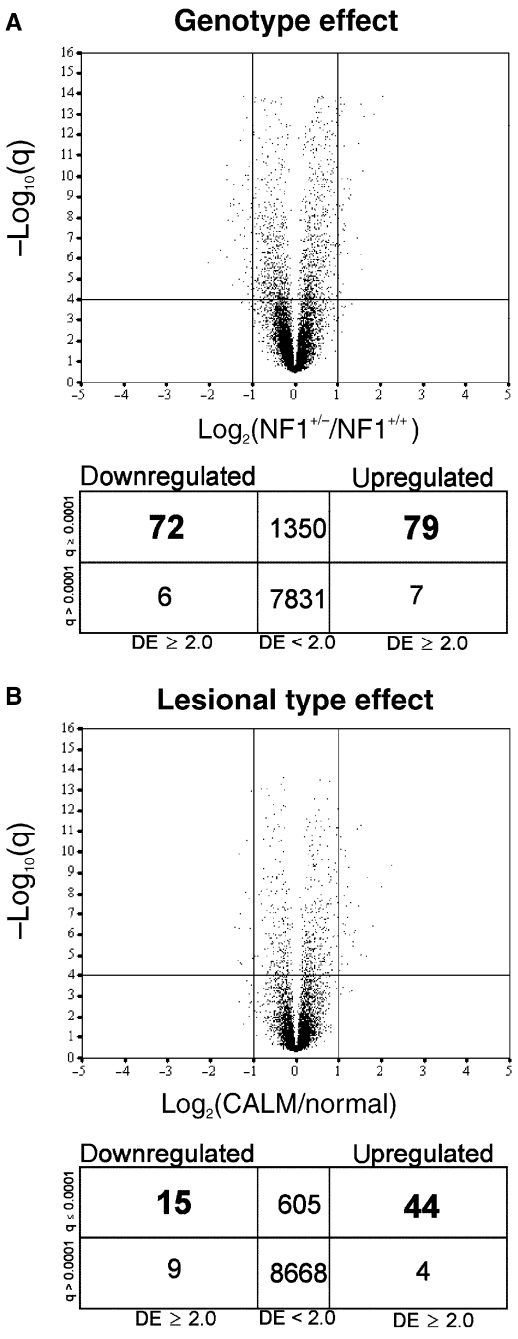

Figure 2. Volcano plots contrasting the significance [– log $_{10}$(q) on the ordinate] and the magnitude of the expression difference ($\log_2$ on the abscissa) for the comparisons between genotypes (A) and skin lesional types (B). Each dot represents one of the 9345 genes. The lowest horizontal line corresponds to the preset false discovery acceptance level of $q = 0.0001$ ($-\log_{10} = 4$). The two vertical lines at $x = 1$ and $x = -1$ demarcates the twofold magnitude of difference. The number of modulated ESTs in each section of the volcano plot are listed in the table below the plot; transcripts for which the two methods (statistical significance and significance as determined by the twofold change criterion) are in agreement are represented in bold typeface.

Table 1. Number (%) of differentially modulated genes

	Upregulated			Downregulated		
	Total	Known	Unknown	Total	Known	Unknown
Genotype[a] (NF1$^{+/-}$ vs NF1$^{+/+}$)	72 (52.6)	68 (49.6)	4 (2.9)	65 (47.4)	62 (45.3)	3 (2.1)
Skin lesional type[b] (CALM skin vs normal skin)	37 (72.5)	35 (68.6)	2 (3.9)	14 (27.5)	14 (27.5)	0 (0.0)
Interaction[c] (NF1$^{+/-}$ × CALM)	183 (39.3)	175 (37.6)	8 (1.7)	282 (60.7)	265 (57.0)	17 (3.7)

[a]A total of 137 genes are modulated at a threshold of DE ≥ twofold for transcripts from NF1$^{+/-}$ melanocytes compared with NF1$^{+/+}$ melanocytes.
[b]Fifty-one genes are modulated at a threshold of DE ≥ twofold for transcripts of hyperpigmentary CALM skin melanocytes compared with normally pigmented skin melanocytes.
[c]A total of 465 genes are modulated at a threshold of DE ≥ twofold for transcripts from a *NF1*$^{+/-}$ genotypic background when CALM skin melanocytes are compared to normal skin melanocytes.
Modulated genes were selected at significance level of $q \leq 0.0001$. Percentages relate to the total number of modulated genes per effect.

included tweety homolog 2 (Drosophila) (*TTYH2*); casein kinase 1, delta (*CSNK1D*); neuronal pentraxin I (*NPTX1*); hematological and neurological expressed 1 (*HN1*); DEAD (Asp-Glu-Ala-Asp) box polypeptide 48 (*DDX48*). In cytoband 19p13.2 some other interesting genes were found, such as growth differentiation factor 15 (GDF15); bone marrow stromal cell antigen 2 (*BST2*); calreticulin (*CALR*); zinc finger protein 14 (KOX 6) (ZNF14). Four genes of the 17q11.2 band containing the *NF1* gene locus were differentially modulated: mitogen-activated protein kinase kinase 3 (*MAP2K3*); RAB34, member RAS oncogene family (*RAB34*); myosin ID (*MYOID*); LIM and SH3 protein 1 (*LASP1*). Further studies aimed at a more detailed analysis of the link between certain genetic regions (and candidate genes) and specific features of NF1 might be of interest for molecular-based (sub)characterization of disease features.

Biological process discovery of differentially modulated genes

Appendix S4, S5 and S6 (see Supplementary material) show significant biological processes for genotype, skin lesional type and genotype × skin lesional type interaction effect, respectively.

Genes involved in regulation of cell proliferation and cell maintenance

The *NF1* gene product, neurofibromin, is a tumor suppressor protein as its primary well-known function is the stimulation of the intrinsic GTPase activity of the oncogene Ras, converting the latter from a GTP-bound, active to a GDP-bound, inactive state. It is therefore not surprising that several genes are involved in regulating cell proliferation. In *NF1*$^{+/-}$ melanocytes, cyclin D1 (*CCND1*) is upregulated compared with *NF1*$^{+/+}$ melanocytes. It has been shown in primary rat Schwann cell cultures that the *Nf1* gene product mediates an antagonizing effect on cAMP and cyclin D1 expression (Kim et al., 2001). Elevated levels of cAMP were even observed in *Nf1* null mice Schwann cells and correlated with increased cyclin D1 expression. The observation in our study that the proto-oncogene cyclin D1, which is a regulator of G1 phase cell cycle progression, is upregulated in human *NF1*$^{+/-}$ melanocytes (sharing a common developmental neural crest origin with Schwann cells), is very interesting in light of the their known hyperproliferative growth in NF1 patients (Ingram et al., 2000). The link between elevated cAMP levels and reduced neurofibromin expression is striking, as it has been demonstrated in Drosophila melanogaster and in mice that neurofibromin can modulate the activity of adenylyl cyclase (Guo et al., 2000; Tong et al., 2002). Some early response genes like v-fos FBJ murine osteosarcoma viral oncogene homolog (*FOS*) and v-maf musculoaponeurotic fibrosarcoma oncogene homolog (avian) (*MAF*) are downregulated while immediate early response 3 (*IER3*) is upregulated in *NF1*$^{+/-}$ (CALM) melanocytes. Two receptor tyrosine kinases are repressed in *NF1*$^{+/-}$ (CALM) melanocytes: the type III receptor tyrosine kinase v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog (*KIT*), important in neural crest development, survival and cell migration, and the c-mer proto-oncogene tyrosine kinase (*MERTK*) (Graham et al., 1994). Interestingly, *MERTK* encodes the receptor of growth arrest specific 6 (*GAS6*), which is thought to be involved in the stimulation of cell proliferation (Chen et al., 1997). GAS6 was found to be a stimulatory factor for human Schwann cell growth (Li et al., 1996). In this report, *GAS6* shows downregulated expression in CALM melanocytes compared with normal skin melanocytes. The G-protein coupled endothelin receptor B (*EDNRB*) is downregulated in *NF1*$^{+/-}$ (CALM) melanocytes. It was found that mutations in the genes encoding endothelin receptor-B (EDNRB) and its ligand endothelin-3 (EDN3) affect the development of neural crest-derived melanocytes during the crucial melanoblast migratory phase. EDNRB-mediated signaling is required for the terminal migration of melanoblasts but this pathway is

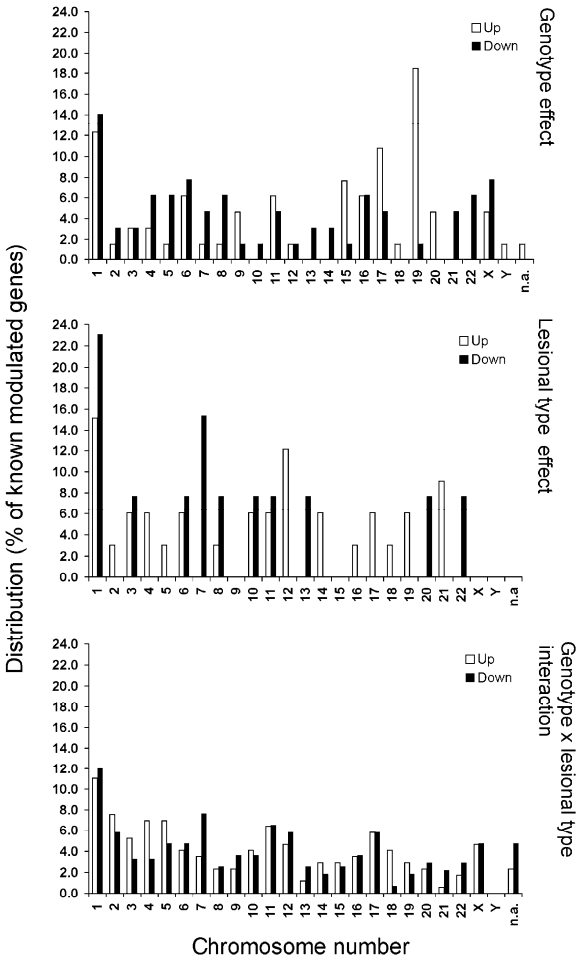

Figure 3. Chromosomal distribution of modulated known genes (genotype effect, skin lesional type effect and genotype × skin lesional type interaction). This graph shows the evaluation of the percentages of modulated genes to the total number of modulated known genes for each chromosome (white bars = upregulated genes; black bars = downregulated genes). The distribution of modulated known genes is shown for genotype effect, lesional type effect and genotype × lesional type interaction effects. n.a: not available (these genes are not yet cytogenetically mapped).

not required for their survival (Lee et al., 2003). As disregulation of melanoblast migration and, hence unequal melanocyte distribution in the epidermis, has been proposed as a results of the haploinsufficient expression of neurofibromin and as a potential explanation in CALM etiopathology (Kemkemer et al., 2002), the effect of *NF1* heterozygosity on *EDNRB* expression could be an important focus point in unraveling the etiopathology of CALMs.

It has been shown that functional neurofibromin levels are dynamically regulated by the ubiquitin-proteasome pathway (Cichowski et al., 2003). Several components of the ubiquitin mediated proteolytic system show differentially modulated gene expression in $NF1^{+/-}$ (CALM) melanocytes: ubiquitin carboxyl-terminal esterase L1 (ubiquitin thiolesterase) (*UCHL1*); ubiquitin-conjugating enzyme E2M (UBC12 homolog, yeast) (*UBE2M*); ubiquitin-conjugating enzyme E2Q (putative) (*UBE2Q*); ubiquitin-like 3 (*UBL3*). Increasing evidence indicates that accumulation of aberrant or misfolded proteins, ubiquitin-proteasome system dysfunction or failure of axonal and dendritic transport represent unifying events in many (slowly) progressive neuro(degenerative) disorders (Bossy-Wetzel et al., 2004). The molecular and functional analysis of the proteasome pathway and its role in the regulation of neurofibromin levels could be an interesting investigative route in order to elucidate the etiopathology of NF1.

Genes involved in cellular adhesion, cytoskeleton, cell motility and extracellular matrix

Several genes involved in cellular adhesion and (actin-)cytoskeletal organization and biosynthesis show modulated expression. Most genes in this category are functional and/or structural components of cell proliferation. Genes encoding extracellular matrix (ECM) proteins and components, such as fibronectin-1 (*FN1*) and laminin, gamma 1 (*LAMC1*), show upregulated expression in $NF1^{+/-}$ (CALM) melanocytes. Indeed, in early melanocyte cell culture experiments from patients with NF1, the ECM contained fibronectin. However, no laminin was detectable because of antibody sensitivity (Kaufmann et al., 1989). The gene encoding syndecan binding protein (syntenin) (*SDCBP*), which binds to the cytoplasmic tail of syndecan (Grootjans et al., 1997), is repressed in $NF1^{+/-}$ melanocytes compared with $NF1^{+/+}$ melanocytes. This is an important observation in light of the known interaction of neurofibromin with syndecan where it has been suggested that this bipartite protein complex is a mechanism to localize neurofibromin to specialized domains of the plasma membrane (Hsueh et al., 2001), perhaps mediating signaling to the cytoskeleton. Another interesting gene, nidogen/entactin (*NID*), encodes a matrix protein which is a ubiquitous component of basement membrane zones underneath developing epithelia of most of the major organ systems (Miosge et al., 2001). Nidogen-1 is thought to be central in the assembly processes, connecting the basement membrane networks formed by collagen type IV and laminins. It has been shown that the interaction between two basement membrane proteins, nidogen and laminin C1, is crucial for the regulation of basement membrane formation in skin-organotypic cocultures (Breitkreutz et al., 2004). The nidogen encoding gene has been proposed as melanocyte biomarker (Dooley et al., 2003) and, together with laminin C1 (*LAMC1*), they are upregulated in $NF1^{+/-}$ melanocytes. This could point towards a potential (indirect) regulatory role of the *NF1* gene product in mediating basement membrane formation. Several members of the semaphorin gene family [semaphorin 3C (*SEMA3C*); semaphorin 4C

(*SEMA4C*); semaphorin 6A (*SEMA6A*)], encoding transmembrane and secreted proteins involved in neurogenesis, axon guidance and cell migration (Ginzburg et al., 2002), are downregulated in $NF1^{+/-}$ (CALM) melanocytes. Interestingly, the gene encoding plexin C1 (*PLXC1*) which is a member of the transmembrane plexin family of putative semaphorin receptors, is also repressed in $NF1^{+/-}$ (CALM) melanocytes. It has been described that the semaphorin/plexin signaling system has a role in epidermal cell positioning and adhesion during epidermal morphogenesis and nervous system development in *C. elegans* (Fujii et al., 2002; Ginzburg et al., 2002). The possible role of this signaling complex in epidermal cell positioning could be an additional element to a proposed model of CALM development in NF1 patients. In this model, it was postulated that an embryological migration problem of melanocyte precursors, because of *NF1* haploinsufficiency, causes a disruption in dendrite formation, and hence affects their migration, leading to an unequal epidermal distribution and positioning of melanocytes in lesional CALM epidermis (Kemkemer et al., 2002).

Genes involved in immune response

Several differentially modulated genes are involved in the immune response, among which several members of the major histocompatibility complex (MHC) family (major histocompatibility complex, class I, A; major histocompatibility complex, class I, B; major histocompatibility complex, class I, C and major histocompatibility complex, class I, E). It has been suggested that mutated Ras activates mechanisms that favor tumor growth and modulation of tumor-specific immune responses. Several new functions of Ras, such as downregulation of major histocompatibility complex molecules, have been uncovered in the last decade (Weijzen et al., 1999). In light of the function of the *NF1* gene product (neurofibromin) as negative regulator of Ras activity, it is not surprising that haploinsufficient expression in *NF1* heterozygous patient cells induces the expression of (benign tumor) specific immune response genes.

Confirmation of microarray results by quantitative real-time PCR

To substantiate the results of the microarray data, we performed quantitative real-time PCR analysis (q-RT-PCR) to analyze the mRNA levels of seven genes across the experimental samples. For all the genes, the results of the q-RT-PCR analysis were in agreement with those from the microarray data (Figure 4). A subset of mainly transcriptional regulators (*SOX10*, *LEF1*, *MITF*), the receptor tyrosine kinase *KIT*, the melanocyte marker *DCT*, *FOS* and *SNAI2* were verified. A similar confirmation was carried out on *MITF*, *DCT*, *TRP1* and *SNAI2* gene expression in other NF1 and control patient melanocytes (Table 2).

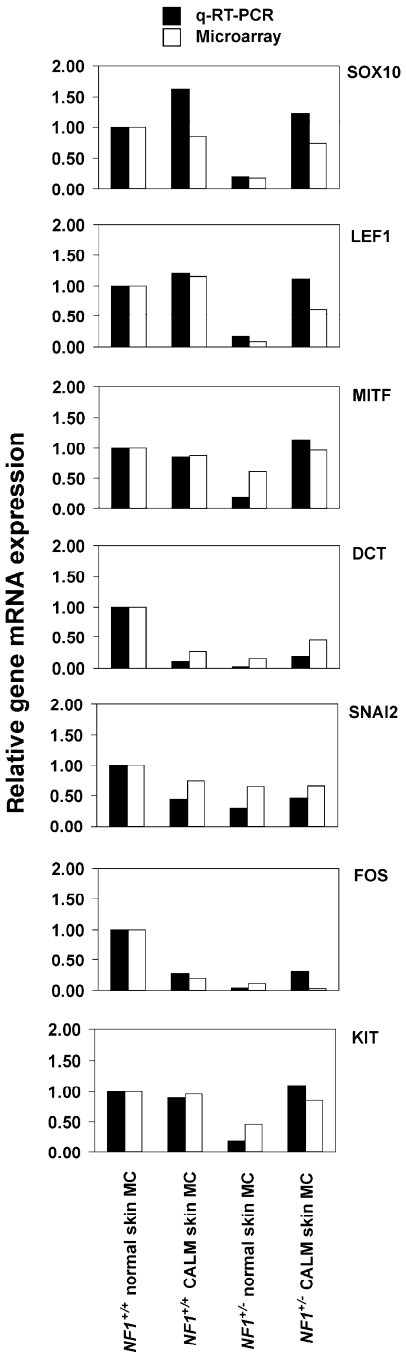

Figure 4. Verification of microarray results by quantitative real-time PCR. q-RT-PCR confirms the expression of a specific subset of genes. *SOX10*, SRY (sex determining region Y)-box 10; *LEF1*, lymphoid enhancer-binding factor 1; *MITF*, microphthalmia-associated transcription factor; *DCT*, dopachrome tautomerase; *SNAI2*, snail homolog 2 (Drosophila); *FOS*, v-fos FBJ murine osteosarcoma viral oncogene homolog; *KIT*, v kit Hardy Zuckerman 4 feline sarcoma viral oncogene homolog. Black bars represent q-RT-PCR results, white bars represent microarray results. Values are adjusted and normalized to the $NF1^{+/+}$ normal skin melanocyte group.

Table 2. Quantitative real-time PCR in other cultured patient melanocytes

Gene	Group[a]	Microarray results	q-RT-PCR
MITF	$NF1^{+/+}$ normal skin MC (n = 3)	1.00	1.00 ± 0.25
	$NF1^{+/+}$ CALM skin MC (n = 2)	0.87	0.73 ± 0.01
	$NF1^{+/-}$ normal skin MC (n = 2)	0.61	0.60 ± 0.04
	$NF1^{+/-}$ CALM skin MC (n = 1)	0.97	0.91 ± 0.00
DCT	$NF1^{+/+}$ normal skin MC (n = 3)	1.00	1.00 ± 0.21
	$NF1^{+/+}$ CALM skin MC (n = 2)	0.27	0.39 ± 0.01
	$NF1^{+/-}$ normal skin MC (n = 2)	0.16	0.15 ± 0.04
	$NF1^{+/-}$ CALM skin MC (n = 1)	0.45	0.18 ± 0.00
TRP1	$NF1^{+/+}$ normal skin MC (n = 3)	1.00	1.00 ± 0.21
	$NF1^{+/+}$ CALM skin MC (n = 2)	0.97	0.64 ± 0.05
	$NF1^{+/-}$ normal skin MC (n = 2)	0.72	0.49 ± 0.01
	$NF1^{+/-}$ CALM skin MC (n = 1)	0.96	0.87 ± 0.00
SNAI2	$NF1^{+/+}$ normal skin MC (n = 3)	1.00	1.00 ± 0.15
	$NF1^{+/+}$ CALM skin MC (n = 2)	0.74	0.66 ± 0.09
	$NF1^{+/-}$ normal skin MC (n = 2)	0.64	0.30 ± 0.02
	$NF1^{+/-}$ CALM skin MC (n = 1)	0.66	0.27 ± 0.00

[a]Number of patients included per melanocyte group is depicted as n. The q-RT-PCR results are mean ± SEM. Microarray results are not mean ± SEM as only one patient per group was included.

Cis-regulatory network mediating transcription of *DCT* gene: role for the *NF1* gene product?

How neurofibromin regulates the expression of melanocyte differentiation markers during melanocyte development, migration and survival is currently unknown. How haplo-insufficient expression of neurofibromin in NF1 patients affects the melanocyte lineage is even a bigger mystery. It has been suggested that neurofibromin is involved in the regulation of tyrosinase (*TYR*) and dopachrome tautomerase (*DCT*) gene expression by activation of their promotor. Transient cotransfection of a luciferase reporter construct (containing a melanocyte-specific tyrosinase enhancer element) and a full-length rat cDNA representing type I neurofibromin mRNA, showed activation of the tyrosinase promotor in MeWo cells, which are deficient of neurofibromin (Suzuki et al., 1994). A similar experiment proposed a regulatory role for neurofibromin in activation of the *DCT* gene promotor (Suzuki et al., 1998). This activation is possibly caused by indirect effects of neurofibromin on their promotor activity, as neurofibromin was thought to have no nuclear localization (Gregory et al., 1993; Nordlund et al., 1993). However, nuclear staining for neurofibromin has been reported in developing neurons (Li et al., 2001) and differentiating keratinocytes (Koivunen et al., 2000). Recently a functional nuclear localization signal (NLS) was found in exon 43 of neurofibromin, enabling it to shuttle between the nucleus and the cytoplasm (Vandenbroucke et al., 2004). No evidence is present yet as to whether neurofibromin could function as a (direct) transcriptional regulator of gene expression.

A large number of genes encoding transcription factors and transcriptional regulators showed modulated expression in $NF1^{+/-}$ (CALM) melanocytes (Table 3). Most of the genes are involved in DNA-dependent regulation of transcription and comprise several upregulated and downregulated members of the high mobility group (HMG) gene family of transcriptional inducers, such as HMG-box transcription factor 1 (*HBP1*), high mobility group box 2 (*HMGB2*), high mobility group AT-hook 1 (*HMGA1*) and high mobility group 20B (*HMG20B*), which are believed to be associated with highly transcriptionally active chromatine regions. Other HMG-box domain containing transcription factors belong to the SOX (SRY-related HMG-box) family of transcription factors (SOX4, SOX10) which are involved in regulation of embryonic development and cell fate determination (Harley et al., 2003). In our study, both *SOX4* and *SOX10* are downregulated in $NF1^{+/-}$ (CALM) melanocytes. SOX10 is a nucleocytosplasmic shuttleprotein important in neural crest development and peripheral nervous system (PNS) development (Paratore et al., 2001), and mutations have been identified in Waardenburg–Hirschsprung patients (Chan et al., 2003). Interestingly, SOX4 may act as transcriptional regulator after protein complex formation with other proteins, such as syndecan binding protein (syntenin). The latter is involved in cell adhesion and primarily localizes to membrane-associated adherens junctions and focal adhesions, but is also known to affect activation of transcription (Zimmermann et al., 2001). cAMP responsive element binding protein-like 2 (*CREBL2*) is repressed in $NF1^{+/-}$ (CALM) melanocytes. This could be an interesting candidate for an unidentified CRE-like motif binding protein (CLMBP) which has been proposed as transcriptional coactivator, together with the cooperation of MITF-M and lymphoid enhancer-binding factor 1 (LEF1), in the activation of *DCT* expression (Yasumoto et al., 2002). The genes encoding MITF and LEF1 are also downregulated in $NF1^{+/-}$ (CALM) melanocytes. Several studies have focused on MITF and SOX10, which have been found to be crucial for neural-crest derived melanocyte development (Potterf et al., 2001). It has been suggested that the murine early melanocyte differentiation gene *Dct* is a target for transcriptional regulators, such as Mitf and Sox10, and that their functional cooperation activities *Dct* expression and might constitute a cis-regulatory network during melanocyte development (Jiao et al., 2004; Ludwig et al., 2004).

The observation of modulated gene expression of several key transcriptional regulators like *SOX10*, *LEF1* and *MITF* could be an interesting focus point in the elucidation of their potential role in the etiopathogenesis of CALMs. This report demonstrates that haploinsufficient expression of neurofibromin in NF1 melanocytes correlates with downregulation of the melanocyte-specific differentiation marker *DCT* and its transcriptional regulators, such as *SOX10*, *LEF1* and *MITF*. These results could point to a direct/indirect role of the *NF1* gene product on the expression of *DCT*.

We must emphasize that the above results cannot be correlated or compared with the in vivo situation as no

Table 3. Differentially modulated transcription factors sorted according to functional category.

Gene name	Gene symbol	Locus link ID	DE	Effect[a]
Transcription (P = 0.100)				
cAMP responsive element binding protein-like 2	CREBL2	1389	2.64	l_d
Death associated transcription factor 1	DATF1	11083	2.11	G_u
Polymerase (RNA) II (DNA directed) polypeptide E, 25 kDa	POLR2E	5434	2.20	G_u
polymerase (RNA) II (DNA directed) polypeptide L, 7.6 kDa	POLR2L	5441	3.89	l_u
Ras-related GTP binding C	RRAGC	64121	2.05	l_d
Negative regulation of transcription (P = 0.302)				
Prohibitin	PHB	5245	2.15	G_u
Positive regulation of transcription (P = 0.021)				
High mobility group AT-hook 1	HMGA1	3159	2.90/2.25/5.16	G_u/L_d/l_u
Interferon regulatory factor 4	IRF4	3662	2.01	G_d
Regulation of transcription, DNA-dependent (P = 0.075)				
Activating transcription factor 4 (tax-responsive enhancer element B67)	ATF4	468	2.04	L_d
Basic helix-loop-helix domain containing, class B, 3	BHLHB3	79365	4.68	l_d
Calreticulin	CALR	811	2.56	l_d
Death associated transcription factor 1	DATF1	11083	2.11	G_u
Delta sleep inducing peptide, immunoreactor	DSIPI	1831	2.24	l_d
Ets variant gene 5 (ets-related molecule)	ETV5	2119	2.24	L_u
General transcription factor IIA, 1, 19/37 kDa	GTF2A1	2957	2.40	l_u
General transcription factor IIF, polypeptide 2, 30 kDa	GTF2F2	2963	2.00	G_d
High-mobility group AT-hook 1	HMGA1	3159	2.90/2.25/5.16	G_u/L_d/l_u
High-mobility group 20B	HMG20B	10362	2.07	G_u
High-mobility group box 2	HMGB2	3148	2.64	l_u
HMG-box transcription factor 1	HBP1	26959	2.28	l_d
Hypothetical protein FLJ20344	FLJ20344	55634	2.23	l_d
Hypoxia-inducible factor 1, alpha subunit	HIF1A	3091	2.90	l_u
Interferon regulatory factor 4	IRF4	3662	2.01	G_d
Interferon, gamma-inducible protein 16	IFI16	3428	2.51/2.15	G_d/l_d
Interferon-stimulated transcription factor 3, gamma 48 kDa	ISGF3G	10379	2.07	l_d
Lymphoid enhancer-binding factor 1	*LEF1*	*51196*	*1.81*	*G_d*
MAX interacting protein 1	MXI1	4601	2.57	l_d
Microphthalmia-associated transcription factor	*MITF*	*4286*	*4.22*	*l_d*
MYC-associated zinc finger protein (purine-binding transcription factor)	MAZ	4150	2.54	l_d
Nuclear factor I/B	NFIB	4781	2.29	l_d
Nuclear receptor coactivator 5	NCOA5	57727	4.04	l_d
Scaffold attachment factor B2	SAFB2	9667	2.17	G_u
Snail homolog 2 (Drosophila)	*SNAI2*	*6591*	*1.87/1.99*	*G_d/l_d*
SRY (sex determining region Y)-box 4	SOX4	6659	3.30	l_d
Transducin-like enhancer of split 1 (E(sp1) homolog, Drosophila)	TLE1	7088	3.37	l_d
Vitamin D (1,25-dihydroxyvitamin D3) receptor	VDR	7421	2.18	l_u
v-maf musculoaponeurotic fibrosarcoma oncogene homolog (avian)	MAF	4094	2.14/3.22	G_d/l_d
Zinc finger protein 14 (KOX 6)	ZNF14	7561	2.04/2.47	G_d/l_d
Regulation of transcription from Pol I promoter (P = 0.021)				
Polymerase (RNA) II (DNA directed) polypeptide L, 7.6 kDa	POLR2L	5441	3.89	l_u
Transcription from Pol II promoter (P = 0.116)				
ADP-ribosyltransferase (NAD + ; poly (ADP-ribose) polymerase)	ADPRT	142	2.32	l_d
Interferon-stimulated transcription factor 3, gamma 48 kDa	ISGF3G	10379	2.07	l_d
v-maf musculoaponeurotic fibrosarcoma oncogene homolog (avian)	MAF	4094	2.14/3.22	G_d/l_d
Myelin expression factor 2	MYEF2	50804	2.73	l_d
Pirin	PIR	8544	3.02/4.73	G_d/l_d
Polymerase (RNA) II (DNA directed) polypeptide E, 25 kDa	POLR2E	5434	2.20	G_u
Polymerase (RNA) II (DNA directed) polypeptide L, 7.6 kDa	POLR2L	5441	3.89	l_u
Pituitary tumor-transforming 1	PTTG1	9232	3.59	l_u
Regulation of transcription from Pol II promoter (P = 0.453)				
v-fos FBJ murine osteosarcoma viral oncogene homolog	*FOS*	*2353*	*2.21/2.77*	*G_d/l_d*
Lipopolysaccharide-induced TNF factor	LITAF	9516	2.54	l_d
SRY (sex determining region Y)-box 10	*SOX10*	*6663*	*2.27/3.18*	*G_d/l_d*
Spi-B transcription factor (Spi-1/PU.1 related)	SPIB	6689	2.31	l_d

Table 3. Continued

Gene name	Gene symbol	Locus link ID	DE	Effect[a]
Transcription initiation from Pol II promoter (P = 0.035)				
General transcription factor IIA, 1, 19/37 kDa	GTF2A1	2957	2.40	l_u
General transcription factor IIF, polypeptide 2, 30 kDa	GTF2F2	2963	2.00	G_d
MYC-associated zinc finger protein (purine-binding transcription factor)	MAZ	4150	2.54	l_d
Transcription termination from Pol II promoter (P = 0.000)				
MYC-associated zinc finger protein (purine-binding transcription factor)	MAZ	4150	2.54	l_d
Transcription from Pol III promoter (P = 0.036)				
Polymerase (RNA) II (DNA directed) polypeptide L, 7.6 kDa	POLR2L	5441	3.89	l_u
Sjogren syndrome antigen B (autoantigen La)	SSB	6741	2.68	l_u

[a]G, genotype effect, L, skin lesional type effect; I, genotype × skin lesional type interaction. Up- or downregulation is represented by '_u' or '_d', respectively, and is appended to the effect. Genes analysed by q-RT-PCR are depicted in italic type face.

in vivo *NF1* gene expression profiles of (non)lesional melanocytes of NF1 patients exist. This poses a problem regarding our results, because hyperpigmentary CALM spots do show increased melanogenesis. This could be explained by the fact that specific microenvironmental cues, such as paracrine and/or autocrine cytokine networks, diffusion of growth factors or specific cell–cell interactions in the epidermis, could switch the expression of neurofibromin to increased levels in (lesional) skin epidermis of NF1 patients, may be upregulating the expression of melanocytic differentiation markers and resulting in the typical hyperpigmented phenotype. A potential mechanism underlying epidermal hyperpigmentation in CALMs of NF1 patients involves upregulated expression of dermal SCF and HGF (hepatocyte growth factor) resulting in the activation of lesional melanocytes (Okazaki et al., 2003). Equal and even increased neurofibromin expression has been observed in epidermal skin sections of NF1 patients compared with normal healthy patients (Malhotra and Ratner, 1994; own observations). The melanocytes used in our experiment were deprived of SCF 72 h before RNA isolation. This was carried out because in primary human melanocytes SCF elicits a significant fluctuation of *NF1* gene expression that is restored to 'baseline levels' after 72 h (own observations). SCF is an important melanocyte mitogen and was added to our melanocytes because it substantially improved the proliferation and survival of $NF1^{+/-}$ melanocytes, and especially $NF1^{+/-}$ CALM melanocytes. These melanocytes are known to have a very slow and impaired cell growth (with a seriously increased population doubling time) under in vitro culture conditions (Kaufmann et al., 1989; own observation). Our primary goal was to detect gene expression differences in melanocytes because of *NF1* heterozygosity and to find potential clues on the etiopathology of CALM spots, while we were not focused on the influence of physiological cytokines such as SCF.

The results presented have been obtained using the cDNA microarray platform. A recent paper has shown that the quality of gene expression data from this type of array has lower correlations across experiments in comparison with commercial platforms (especially the oligo arrays), and that the discrepancies are mainly because of higher levels of noise, clone errors, annotation problems, genome coverage, etc. (Järvinen et al., 2004). These intrinsic problems associated with analysis of cDNA arrays may limit the interpretation of their results. However, another recent publication (Allemeersch et al., 2005) demonstrated that cDNA arrays have the same specificity and sensitivity than the commercial oligo arrays from Affimetrix and Agilent. In addition, we believe that the clone errors on the VIB Human Array are less than 10% because they were sequenced verified by RZPD (see Materials and methods) and our RT-PCR validations gave 100% correlation with the microarray data, at least on a restricted and most relevant subset of the differentially expressed genes (Figure 4; Table 2).

In addition, we included only a restricted number of patients (one NF1 and one control patient) that forces us to postulate that we cannot rule out genetic diversity across our two patients, nor do the applied statistics. However, by considering differentially expressed genes with at least a twofold change (up or down), we have attempted to minimize small background differential gene expression because of (interpatient) genetic variation. However, we do not deny that twofold changes can occur based on differences in genetic background. While there are dangers in using cDNA arrays, the results that we have obtained with a restricted set of patients using real-time PCR appear to confirm the cDNA array data, at least on a restricted subset of genes.

In conclusion, this study addresses for the first time the effect of a heterozygous *NF1* gene mutation on the expression of the human melanocyte transcriptome. This study generated several potentially interesting candidate genes that could be helpful in the future investigation of the etiopathology of benign as well as malignant tumors in NF1 patients. It will be of great interest to further analyze the potential role of transcriptional regulators in the

expression of melanocyte-specific differentiation markers. Specifically their disregulated expression in pigmentary skin disorders deserves much attention. This report describes possible new investigative pathways by presenting putative transcriptional regulatory candidates, of which disregulated expression could be explained as part of the effect of *NF1* gene mutation. A possible link between the aberrant expression of a mutated *NF1* gene, transcriptional regulation of target genes and micro-environmental factors in vivo, might be helpful in the elucidation of the etiopathology of hyperpigmentary CALMs in NF1 patients.

Materials and methods

Cell culture and total RNA isolation

A skin biopsy of normally pigmented and hyperpigmented CALM skin of a NF1 patient and a healthy control patient was taken using 5 mm punch biopsy excision, brought in 10% RAID solution and incubated overnight at 4°C before subsequent processing. Primary epidermal melanocyte cultures were established as described previously (Naeyaert et al., 1991). Briefly, skin biopsies were incubated overnight at 4°C in 10% dispase II (Boehringer Mannheim, Mannheim, Germany) to separate the epidermal layer (with melanocytes anchored to the basal membrane) from the underlying dermis. Melanocytes were cultured in Ham's F10 medium (Gibco, Invitrogen Ltd, Paisley, UK) supplemented with 2.5% fetal calf serum (FCS), 1% Ultroser, 5 ng/ml basic fibroblast growth factor (bFGF), 10 ng/ml endothelin-1 (ET-1), 0.33 nM cholera toxin (CT), 0.033 mM isobutyl-methyl-xanthine (IBMX), 5.3 nM 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) and 20 ng/ml stem cell factor (SCF). Seventy-two hours before cell harvesting, melanocytes were maintained in SCF depleted culture medium. Cells were harvested between passage 2 and 4. Total RNA was isolated and purified using the QIAGEN RNEasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

Construction of microarrays

The VIB Human 21K I cDNA High Density microarray (VIB MicroArray Facility, Leuven, Belgium) consists of 20,988 cDNA fragments (ESTs) and 384 controls (calibration and negative control spots), printed on type VII silane-coated slides (Amersham Biosciences, Little Chalfont, UK). The clone set includes 5421 cDNAs from the Incyte clone collection (Incyte Genomics, Palo Alto, CA, USA), 15 424 cDNAs from the RZPD clone collection (RZPD; Deutsches Ressourcenzentrum für Genomforschung GmbH, Berlin, Germany) and 143 intrinsic additional clones. Using the most recent Clone Information Update (CIU) HS6, the VIB Human 21K I cDNA microarray harbors ~13 850 unique, fully annotated genes. A complete description of the array content and the printing procedures can be downloaded from ArrayExpress with accession number: A-MEXP-146 (http://www.ebi.ac.uk/arrayexpress).

RNA amplification and labeling

Antisense RNA was amplified using in vitro transcription previously described in Puskás et al. (2002). In brief, 5 μg of total RNA was reverse transcribed to double stranded cDNA with a oligo(dT) +T7 promotor. After purification of the double stranded cDNA, RNA was synthesized by the AmpliScribe T7 high-yield transcription kit (Epicentre Technologies, Madison, WI, USA) and 5 μg of the aRNA was labeled by reverse transcription with Cy3-dCTP or Cy5-dCTP (Amersham Biosciences).

Array hybridization and post-hybridization processes

Arrays were hybridized with 40 pmol incorporated Cy5 or Cy3 mixed in 210 μl hybridization solution (cat. no. RPK0325; Amersham BioSciences) at 45°C using an Automated Slide Processor (ASP) (Amersham BioSciences). Post-hybridization washing was performed in 1X SSC, 0.1% SDS, followed by 0.1X SSC, 0.1% SDS and 0.1X SSC. The complete ASP program can be downloaded from http://www.microarrays.be (/servicemainframe.htm#protocolscap) or via ArrayExpress with accession number: P-MEXP-581 (http://www.ebi.ac.uk/arrayexpress).

Scanning and data analysis

Slides were scanned using a Generation III scanner (Amersham Biosciences), with wavelength settings at 532 nm (Cy3 signal) and 635 nm (Cy5 signal). Image analysis was performed with ArrayVision (Imaging Research Inc., St Catharines, Ontario, Canada). All analyses were performed on artifact-removed, non-background-subtracted, less-transformed log base two fluorescence intensity measurements. Only ESTs having a positive signal for each genotype/skin lesional type/replicate combination were taken into further analysis.

Two-step mixed model analysis of variance (Wolfinger et al., 2001) was performed with the statistical software package GenStat 7.0 for Windows (VSN International Ltd, Herts, UK). Each of the eight hybridization samples was subjected to a linear normalization with the model $y_{ij} = \mu + \text{array}_i + \text{printtip}_j + \text{array} \times \text{printtip}_{ij} + \text{residual}_{ij}$, where y_{ij} are the $\log_2$-transformed expression intensities, i ($i = 1\ldots8$) specifies the random array effects and j ($j = 1\ldots24$) specifies the printtip. The residuals from this model were then analyzed in separate mixed models by probe using a model of the form $\text{residual}_{ijklm} = \mu + \text{replicate}_k + \text{dye}_j + \text{genotype}_l + \text{skin lesional type}_m + \text{genotype} \times \text{skin lesional type}_{lm} + \text{array}_i + \text{error}_{jklm}$, where k specifies the biological replicate, l specifies the genotype ($NF1^{+/-}$ or $NF1^{+/+}$), m is the skin lesional type (normal pigmented skin or hyperpigmented CALM skin), and array is the only random effect. The significance of each of the two main effects genotype and skin lesional type as well as the contrasts of the two skin lesional types within each genotype was then determined using the Wald statistic. We used the recently introduced q-value as a 'false discovery rate' (FDR)-based measure of significance for genome-wide studies (Storey and Tibshirani, 2003). The q-values were computed directly from the calculated P-values by using the software qvalue (http://genomine.org/qvalue/); default parameter settings were used to estimate π_0 (proportion of features that are truly null). Only those genes for which the magnitude of the genotype, skin lesional type and the genotype × skin lesional type interaction effect was greater than or equal to twofold and with a q-value ≤ 0.0001 were selected for further analysis.

Quantitative real-time PCR analysis

Analysis of relative gene expression was performed on a subset of modulated genes to confirm microarray results. Quantitative real-time PCR was performed using the ABI Prism 7000 and the $2^{-\Delta\Delta CT}$ method for relative gene expression (Vandesompele et al., 2002). For each gene, specific primer pairs were designed using Primer Express 2.0 (Applied Biosystems, Lennik, Belgium). Primers used were *SOX10* (forward: 5′-AAC GTG GAC ATT GGT GAG ATC A-3′; reverse: 5′-GGT CCA ACT CAG CCA CAT CA-3′), *LEF1* (forward: 5′-CAT GCG GTC CAT CCT CTC A-3′; reverse: 5′-CGG GTG TGA TCC TGG AGA AA-3′), *SNAI2* (forward: 5′-ATG AGG AAT CTG GCT GCT GT-3′; reverse: 5′-CAG GAG AAA ATG CCT TTG GA-3′), *MITF*

(forward: 5′-CGG CAG CAG GTA AAG CAG TAC-3′; reverse: 5′-TGG CAA GCT CAG GAC TTG GT-3′), *FOS* (forward: 5′-CCA AGC GGA GAC AGA CCA A-3′; reverse: 5′-TTT CCT TCT CCT TCA GCA GGT T-3′), *KIT* (forward: 5′-CTG ATC CGG GCT TTG TCA A-3′; reverse: 5′-CAT TCA TTC TGC TTA TTC TCA TTC G-3′), *DCT* (5′-GGA CTC TGG ATT CTC AAG TGA TGA G-3′; reverse: 5′-GGA TCA TTG GCG GCT GAA-3′) and *TRP1* (forward: 5′-CAA TGG CGA GTG GTC TGT GA-3′; reverse: 5′-TCC TCG GTG CTG TTA CAA AGT G-3′). Appropriate internal control genes, used to normalize for differences in the amount of cDNA added to the reactions, were selected using Genorm. We used the geometric mean of three housekeeping genes for more accurate normalization of our expression data (Vandesompele et al., 2002). The primers were validated for use of the $2^{-\Delta\Delta CT}$ method by making a dilution series of cDNA over a 1000-fold range. The dilution plot showed that the absolute value of the log cDNA dilution versus ΔΔCT slope was close to zero, meaning that amplification efficiencies of the target and reference genes are similar and ΔΔCT calculation can be performed. RNA was isolated using the RNeasy Mini Kit (Qiagen) followed by DNase treatment (Promega, Leiden, The Netherlands). Two μg of DNAse-treated RNA was used to synthesize cDNA with Superscript II RT-enzyme and random hexamers according to the manufacturer's guidelines (Invitrogen, Merelbeke, Belgium). Two step RT-PCR SYBR green assays were performed using a 25 μl mixture containing 12.5 μl 2 × SYBR green PCR mastermix (Applied Biosystems), 5 μl of cDNA template, 300 nM of forward and reverse primer and 4.5 μl RNase free water (Sigma, Bornem, Belgium). The cycling conditions comprised 2 min at 50°C, 10 min of polymerase activation at 95°C and 40 cycles at 95°C for 15 s and 60°C for 60 s. A dissociation curve from 60°C to 95°C was performed after each run to exclude primer-dimer formation.

Functional classification and biological theme discovery

Gene symbols for each of the significantly modulated ESTs (representing known genes) and for the full set of known genes on the microarray were imported into Onto-Express™ (Khatri et al., 2002) (http://vortex.cs.wayne.edu/projects.htm), which was used to search for gene ontology (GO) terms related to known biological processes of these genes and to correlate identified genes with their functional characteristics. A (corrected) significance P-value was assigned to the frequency with which genes were identified in a particular category, based on the total number of genes in that category and represented on the microarray. We used a nominal significance level of 0.05.

Compliance

The microarray data files corresponding to the eight comparative hybridizations are uploaded to the European Bioinformatics Institute's ArrayExpress public data repository at The experiment's accession number is: E-MEXP-258 (http://www.ebi.ac.uk/arrayexpress/).

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Supplementary material

The following supplementary material is available for this article online at www.blackwell-synergy.com:

Appendix S1. Table listing full set of differentially modulated genes due to genotype effect ($NF1^{+/-}$ melanocytes versus $NF1^{+/+}$ melanocytes).

Appendix S2. Table listing full set of differentially modulated genes due to skin lesional type effect (CALM skin melanocytes versus normal skin melanocytes).

Appendix S3. Table listing full set of differentially modulated genes due to genotype x skin lesional type interaction.

Appendix S4. Table listing the biological process categories of differentially modulated genes due to genotype effect.

Appendix S5. Table listing the biological process categories of differentially modulated genes due to lesional type effect.

Appendix S6. Table listing the biological process categories of differentially modulated genes due to gentotype x skin lesional type interaction.

APPENDIX S1 Table listing full set of differentially modulated genes due to genotype effect (NF1+/- versus NF1+/+ melanocytes)

Known / unknown	Gene name	Gene symbol	Accession number	LocusLink ID	DE	Up / down	Chromosome	Cytoband
Known Gene	24-dehydrocholesterol reductase	DHCR24	BC011659	1718	2,17	up	1	1p33-p31.1
Known Gene	A kinase (PRKA) anchor protein (yotiao) 9	AKAP9	NM_147171	10142	2,33	up	7	7q21-q22
Known Gene	adaptor-related protein complex 2, sigma 1 subunit	AP2S1	BM809524	1175	2,00	up	19	19q13.2-q13.3
Known Gene	aldolase A, fructose-bisphosphate	ALDOA	AK098778	226	2,22	up	16	16q22-q24
Known Gene	apolipoprotein E	APOE	AJ249921	348	2,90	up	19	19q13.2
Known Gene	ATPase, Na+/K+ transporting, alpha 1 polypeptide	ATP1A1	NM_000701	476	2,09	up	1	1p21
Known Gene	B-cell receptor-associated protein 31	BCAP31	BM466989	10134	2,14	up	X	Xq28
Known Gene	chondroitin sulfate proteoglycan 4 (melanoma-associated)	CSPG4	X96753	1464	2,07	up	15	15q23
Known Gene	chromosome 9 open reading frame 86	C9orf86	NM_024718	55684	2,26	up	9	9q34.3
Known Gene	coatomer protein complex, subunit epsilon	COPE	BQ717470	11316	2,15	up	19	19p13.11
Known Gene	cyclin D1 (PRAD1: parathyroid adenomatosis 1)	CCND1	NM_053056	595	4,00	up	11	11q13
Known Gene	cytochrome b-245, alpha polypeptide	CYBA	AK097127	1535	2,49	up	16	16q24
Known Gene	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, Y-linked	DDX3Y	NM_004660	8653	2,45	up	Y	Yq11
Known Gene	death associated transcription factor 1	DATF1	AB002331	11083	2,11	up	20	20q13.33
Known Gene	diffuse panbronchiolitis critical region 1	DPCR1	AL833738	135656	2,91	up	6	6p21.32
Known Gene	DKFZP434C131 protein	DKFZP434C131	AL117482	25989	4,12	up	15	15q23
Known Gene	dynactin 4	MGC3248	AK126459	84516	3,52	up	16	16p12.3
Known Gene	enolase 1, (alpha)	ENO1	AK090592	2023	2,38	up	1	1p36.3-p36.2
Known Gene	erythrocyte membrane protein band 4.1-like 3	EPB41L3	AL832598	23136	2,20	up	18	18p11.32
Known Gene	eukaryotic translation initiation factor 4 gamma, 1	EIF4G1	NM_182917	1981	3,82	up	3	3q27-qter
Known Gene	follistatin-like 3 (secreted glycoprotein)	FSTL3	BC005839	10272	2,42	up	19	19p13
Known Gene	high mobility group AT-hook 1	HMGA1	BU739829	3159	2,90	up	6	6p21
Known Gene	high-mobility group 20B	HMG20B	AF370429	10362	2,07	up	19	19p13.3
Known Gene	histone 1, H4c	HIST1H4C	NM_003542	8364	2,35	up	6	6p21.3
Known Gene	HMT1 hnRNP methyltransferase-like 2 (S. cerevisiae)	HRMT1L2	BQ876390	3276	2,62	up	19	19q13.3
Known Gene	hypothetical protein FLJ20850	FLJ20850	AK094710	55049	2,83	up	19	19p13.11
Known Gene	immediate early response 3	IER3	BM994398	8870	2,20	up	6	6p21.3
Known Gene	integrin beta 1 binding protein 3	ITGB1BP3	NM_014446	27231	3,19	up	19	19p13.3
Known Gene	integrin, alpha 3 (antigen CD49C, alpha 3 subunit of VLA-3 receptor)	ITGA3	M59911	3675	2,47	up	17	17q21.33
Known Gene	lysophospholipase II	LYPLA2	NM_007260	11313	2,28	up	1	1p36.12-p35.1
Known Gene	major vault protein	MVP	NM_017458	9961	2,26	up	16	16p13.1-p11.2
Known Gene	mannose-P-dolichol utilization defect 1	MPDU1	BM542186	9526	2,82	up	17	17p13.1-p12
Known Gene	mitochondrial ribosomal protein L12	MRPL12	BM557512	6182	2,13	up	17	17q25
Known Gene	Mov10, Moloney leukemia virus 10, homolog (mouse)	MOV10	AK074174	4343	2,16	up	1	1p13.1
Known Gene	myeloid leukemia factor 2	MLF2	BM919311	8079	2,01	up	12	12p13
Known Gene	myosin, light polypeptide 9, regulatory	MYL9	BM701565	10398	3,50	up	20	20q11.23
Known Gene	natriuretic peptide receptor B/guanylate cyclase B (atrionatriuretic peptide receptor B)	NPR2	L13436	4882	2,13	up	9	9p21-p12
Known Gene	N-deacetylase/N-sulfotransferase (heparan glucosaminyl) 1	NDST1	NM_001543	3340	2,09	up	5	5q33.1
Known Gene	N-ethylmaleimide-sensitive factor attachment protein, alpha	NAPA	AK123456	8775	2,00	up	19	19q13.33
Known Gene	peptidylprolyl isomerase B (cyclophilin B)	PPIB	BG288717	5479	2,31	up	15	15q21-q22
Known Gene	plectin 1, intermediate filament binding protein 500kDa	PLEC1	NM_201380	5339	2,02	up	8	8q24
Known Gene	polymerase (RNA) II (DNA directed) polypeptide E, 25kDa	POLR2E	AK122813	5434	2,20	up	19	19p13.3
Known Gene	presenilin enhancer 2	PEN2	BQ222622	55851	2,54	up	19	19q13.13
Known Gene	procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), beta polypeptide (protein disulfide isomerase; thyroid hormone binding protein p55)	P4HB	J02783	5034	2,00	up	17	17q25
Known Gene	prohibitin	PHB	BF676086	5245	2,15	up	17	17q21
Known Gene	protein phosphatase 1, catalytic subunit, alpha isoform	PPP1CA	AK098311	5499	2,01	up	11	11q13
Known Gene	protein regulator of cytokinesis 1	PRC1	NM_003981	9055	2,01	up	15	15q26.1
Known Gene	proteolipid protein 2 (colonic epithelium-enriched)	PLP2	BF214130	5355	2,02	up	X	Xp11.23
Known Gene	ras homolog gene family, member C	RHOC	BC009177	389	2,20	up	1	1p13.1
Known Gene	regulator of G-protein signalling 12	RGS12	NM_002926	6002	2,02	up	4	4p16.3
Known Gene	regulatory solute carrier protein, family 1, member 1	RSC1A1	NM_006511	6248	2,22	up	1	1p36.1
Known Gene	ribosomal protein S6 kinase, 90kDa, polypeptide 2	RPS6KA2	AK095751	6196	2,81	up	6	6q27
Known Gene	S100 calcium binding protein A16	S100A16	NM_080388	140576	2,73	up	1	1q21
Known Gene	scaffold attachment factor B2	SAFB2	D50928	9667	2,17	up	19	19p13.3
Known Gene	Sec61 alpha 1 subunit (S. cerevisiae)	SEC61A1	NM_013336	29927	2,59	up	3	3q21.3
Known Gene	SH3 domain binding glutamic acid-rich protein like 3	SH3BGRL3	AF466367	83442	2,18	up	1	1p35-p34.3
Known Gene	small EDRK-rich factor 2	SERF2	BM926308	10169	2,97	up	15	15q15.1
Known Gene	small nuclear ribonucleoprotein polypeptides B and B1	SNRPB	BM564070	6628	3,73	up	20	20p13
Known Gene	SMC2 structural maintenance of chromosomes 2-like 1 (yeast)	SMC2L1	AL833191	10592	2,04	up	9	9q31.2
Known Gene	sorting nexin 17	SNX17	NM_014748	9784	2,03	up	2	2p23-p22
Known Gene	splicing factor, arginine/serine-rich 1 (splicing factor 2, alternate splicing factor)	SFRS1	XM_375475	6426	2,15	up	17	17q21.3-q22
Known Gene	thymosin, beta 4, X-linked	TMSB4X	BM994376	7114	2,11	up	X	Xq21.3-q22
Known Gene	TRAF2 binding protein	T2BP	NM_052864	92610	3,30	up	4	4q26
Known Gene	transducer of ERBB2, 1	TOB1	BC031406	10140	2,06	up	17	17q21

APPENDIX S1 (continued)

Known / unknown	Gene name	Gene symbol	Accession number	LocusLink ID	DE	Up / down	Chromosome	Cytoband
Known Gene	transgelin 2	TAGLN2	BQ277068	8407	3,62	up	1	1q21-q25
Known Gene	transmembrane 4 superfamily member 7	TM4SF7	AK126635	7106	2,03	up	11	11p15.5
Known Gene	ubiquitin-conjugating enzyme E2M (UBC12 homolog, yeast)	UBE2M	BM423509	9040	2,43	up	19	19q13.43
Known Gene	zinc finger protein 289, ID1 regulated	ZNF289	NM_032389	84364	2,06	up	11	11p11.2-p11.12
Known Gene	adaptor-related protein complex 1, sigma 2 subunit	AP1S2	BX647433	8905	2,86	down	X	Xp22.31
Known Gene	adducin 3 (gamma)	ADD3	NM_016824	120	2,13	down	10	10q24.2-q24.3
Known Gene	ADP-ribosylation factor-like 7	ARL7	NM_005737	10123	2,11	down	2	2q37.2
Known Gene	beta-site APP-cleaving enzyme 2	BACE2	NM_012105	25825	2,42	down	21	21q22.3
Known Gene	brain expressed X-linked-like 1	BEXL1	XM_043653	56271	2,46	down	X	Xq22.1-q22.3
Known Gene	cadherin 1, type 1, E-cadherin (epithelial)	CDH1	NM_004360	999	2,04	down	16	16q22.1
Known Gene	cadherin 3, type 1, P-cadherin (placental)	CDH3	BC041846	1001	2,14	down	16	16q22.1
Known Gene	carbonic anhydrase XIV	CA14	BM701119	23632	2,13	down	1	1q21
Known Gene	caspase 4, apoptosis-related cysteine protease	CASP4	AL050391	837	2,06	down	11	11q22.2-q22.3
Known Gene	ceroid-lipofuscinosis, neuronal 2, late infantile (Jansky-Bielschowsky disease)	CLN2	NM_000391	1200	2,05	down	11	11p15.5
Known Gene	chromosome 1 open reading frame 29	C1orf29	AL832618	10964	2,97	down	1	1p31.1
Known Gene	chromosome 1 open reading frame 38	C1orf38	AK094833	9473	2,30	down	1	1p35.3
Known Gene	chromosome 5 open reading frame 5	C5orf5	AF251038	51306	2,70	down	5	5q31
Known Gene	dopachrome tautomerase (dopachrome delta-isomerase, tyrosine-related protein 2)	DCT	AJ000503	1638	3,00	down	13	13q32
Known Gene	epidermal growth factor receptor pathway substrate 15	EPS15	NM_001981	2060	2,04	down	1	1p32
Known Gene	galactose-3-O-sulfotransferase 4	GAL3ST4	NM_024637	79690	2,31	down	7	7q22
Known Gene	general transcription factor IIF, polypeptide 2, 30kDa	GTF2F2	NM_024638	2963	2,00	down	13	13q14
Known Gene	glycogenin 2	GYG2	NM_024639	8908	2,01	down	X	Xp22.3
Known Gene	glycoprotein (transmembrane) nmb	GPNMB	NM_024640	10457	3,00	down	7	7p15
Known Gene	GM2 ganglioside activator	GM2A	NM_024641	2760	2,05	down	5	5q31.3-q33.1
Known Gene	guanosine monophosphate reductase	GMPR	NM_024642	2766	2,75	down	6	6p23
Known Gene	hect domain and RLD 5	HERC5	NM_024643	51191	2,06	down	4	4q22.1-q23
Known Gene	HSPC244	MGC13379	NM_024644	51259	2,46	down	11	11q13.1
Known Gene	hypothetical protein FLJ10157	FLJ10157	NM_024645	55083	2,76	down	1	1q44
Known Gene	hypothetical protein FLJ36674	FLJ36674	NM_024646	284040	2,51	down	17	17p11.2
Known Gene	hypothetical protein MGC33607	MGC33607	NM_024647	256309	2,79	down	4	4q35.1
Known Gene	immunoglobulin superfamily, member 3	IGSF3	NM_024648	3321	2,11	down	1	1p13
Known Gene	interferon regulatory factor 4	IRF4	NM_024649	3662	2,01	down	6	6p25-p23
Known Gene	interferon, alpha-inducible protein 27	IFI27	NM_024650	3429	2,31	down	14	14q32
Known Gene	interferon, gamma-inducible protein 16	IFI16	NM_024651	3428	2,51	down	1	1q22
Known Gene	KIAA0367	KIAA0367	NM_024652	23273	2,05	down	9	9q21.31
Known Gene	KIAA1661 protein	KIAA1661	NM_024653	85375	2,31	down	22	
Known Gene	macrophage migration inhibitory factor (glycosylation-inhibiting factor)	MIF	NM_024654	4282	2,66	down	22	22q11.23
Known Gene	MAD2 mitotic arrest deficient-like 2 (yeast)	MAD2L2	NM_024655	10459	2,26	down	1	1p36
Known Gene	major histocompatibility complex, class I, E	HLA-E	NM_024656	3133	2,27	down	6	6p21.3
Known Gene	myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)	MX1	NM_024657	4599	2,84	down	21	21q22.3
Known Gene	myxovirus (influenza virus) resistance 2 (mouse)	MX2	NM_024658	4600	3,95	down	21	21q22.3
Known Gene	NAD(P)H dehydrogenase, quinone 1	NQO1	NM_024659	1728	2,40	down	16	16q22.1
Known Gene	NEDD9 interacting protein with calponin homology and LIM domains	NICAL	NM_024660	64780	2,31	down	6	6q21
Known Gene	oculocutaneous albinism II (pink-eye dilution homolog, mouse)	OCA2	NM_024661	4948	2,35	down	15	15q11.2-q12
Known Gene	peptidylglycine alpha-amidating monooxygenase	PAM	NM_024662	5066	3,74	down	5	5q14-q21
Known Gene	phosphoinositol 3-phosphate-binding protein-2	PEPP2	NM_024663	54477	2,04	down	12	12p12
Known Gene	phospholipase A1 member A	PLA1A	NM_024664	51365	3,12	down	3	3q13.13-q13.2
Known Gene	phosphoprotein associated with glycosphingolipid-enriched microdomains	PAG	NM_024665	55824	2,46	down	8	8q21.12
Known Gene	pirin (iron-binding nuclear protein)	PIR	NM_024666	8544	3,02	down	X	Xp22.31
Known Gene	polymerase (RNA) II (DNA directed) polypeptide F	POLR2F	NM_024667	5435	2,76	down	22	22q13.1
Known Gene	potassium voltage-gated channel, shaker-related subfamily, beta member 2	KCNAB2	NM_024668	8514	2,34	down	1	1p36.3
Known Gene	regulator of G-protein signalling 20	RGS20	NM_024669	8601	2,23	down	8	8q12.1
Known Gene	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3C	SEMA3C	NM_024670	10512	2,01	down	7	7q21-q31
Known Gene	sialyltransferase 1 (beta-galactoside alpha-2,6-sialyltransferase)	SIAT1	NM_024671	6480	2,14	down	3	3q27-q28
Known Gene	solute carrier family 1 (glutamate/neutral amino acid transporter), member 4	SLC1A4	NM_024672	6509	2,14	down	2	2p15-p13
Known Gene	spermidine/spermine N1-acetyltransferase	SAT	NM_024673	6303	3,34	down	X	Xp22.1
Known Gene	splicing factor 3a, subunit 3, 60kDa	SF3A3	NM_024674	10946	2,16	down	1	1p34.2
Known Gene	SRY (sex determining region Y)-box 10	SOX10	NM_024675	6663	2,27	down	22	22q13.1
Known Gene	syndecan binding protein (syntenin)	SDCBP	NM_024676	6386	2,03	down	8	8q12
Known Gene	TBC1 domain family, member 16	TBC1D16	NM_024677	125058	2,00	down	17	17q25.3
Known Gene	ubiquitin carboxyl-terminal esterase L1 (ubiquitin thiolesterase)	UCHL1	NM_024678	7345	2,19	down	4	4p14
Known Gene	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 10 (GalNAc-T10)	GALNT10	NM_024679	55568	2,01	down	5	5q33.2
Known Gene	v-fos FBJ murine osteosarcoma viral oncogene homolog	FOS	NM_024680	2353	2,21	down	14	14q24.3
Known Gene	v-maf musculoaponeurotic fibrosarcoma oncogene homolog (avian)	MAF	NM_024681	4094	2,14	down	16	16q22-q23

APPENDIX S1 (continued)

Known / unknown	Gene name	Gene symbol	Accession number	LocusLink ID	DE	Up / down	Chromosome	Cytoband
Known Gene	WD40 repeat protein interacting with phosphoInositides of 49kDa	WIPI49	NM_024682	55062	2,14	down	17	17q24.2
Known Gene	zinc finger protein 14 (KOX 6)	ZNF14	NM_024683	7561	2,04	down	19	19p13.3-p13.2
Unknown Gene	-	-	NM_024684	-	2,45	up	-	-
Unknown Gene	Homo sapiens transcribed locus	-	NM_024685	-	2,24	up	-	-
Unknown Gene	Homo sapiens transcribed locus	-	NM_024686	-	2,05	up	-	-
Unknown Gene	hypothetical protein LOC163782	-	NM_024687	-	2,22	up	-	-
Unknown Gene	-	-	NM_024688	-	2,00	down	-	-
Unknown Gene	-	-	NM_024689	-	2,56	down	-	-
Unknown Gene	Homo sapiens transcribed locus	-	NM_024690	-	3,09	down	-	-

APPENDIX S2 **Table listing full set of differentially modulated genes due to skin lesional type effect (CALM skin melanocytes versus normal skin melanocytes)**

Known / unknown	Gene name	Gene symbol	Accesion number	LocusLink ID	DE	Up / down	Chromosome	Cytoband
Known Gene	2' 5'-oligoadenylate synthetase 1, 40/46kDa	OAS1	AK123526	4938	3,13	up	12	12q24.1
Known Gene	2'-5'-oligoacenylate synthetase 2, 69/71kDa	OAS2	NM_002535	4939	5,25	up	12	12q24.2
Known Gene	2'-5'-oligoacenylate synthetase 3, 100kDa	OAS3	AB044545	4940	2,44	up	12	12q24.2
Known Gene	ATX1 antioxidant protein 1 homolog (yeast)	ATOX1	AK128210	475	2,05	up	5	5q32
Known Gene	B aggressive lymphoma gene	BAL	AF307338	83666	2,17	up	3	3q13-q21
Known Gene	beta-site APP-cleaving enzyme 2	BACE2	NM_012105	25825	2,02	up	21	21q22.3
Known Gene	BMP and activin membrane-bound inhibitor homolog (Xenopus laevis)	BAMBI	NM_012342	25805	2,40	up	10	10p12.3-p11.2
Known Gene	bone marrow stromal cell antigen 2	BST2	BU739064	684	4,70	up	19	19p13.2
Known Gene	caspase 1, apoptosis-related cysteine protease (interleukin 1, beta, convertase)	CASP1	NM_033292	834	2,05	up	11	11q23
Known Gene	chromosome 1 open reading frame 14	C1orf14	BC026084	81626	2,02	up	1	1q25
Known Gene	chromosome 1 open reading frame 29	C1orf29	AL832618	10964	2,23	up	1	1p31.1
Known Gene	DNase II-like acid DNase	DLAD	NM_021233	58511	2,43	up	1	1p22.3
Known Gene	ets variant gene 5 (ets-related molecule)	ETV5	NM_004454	2119	2,24	up	3	3q28
Known Gene	hect domain and RLD 5	HERC5	NM_016323	51191	2,53	up	4	4q22.1-q23
Known Gene	hypothetical protein DKFZp434F0318	DKFZP434F0318	BC042478	81575	2,10	up	12	12p13.2
Known Gene	hypothetical protein MGC12538	MGC12538	AK092215	84832	2,06	up	1	1q23.3
Known Gene	interferon induced transmembrane protein 1 (9-27)	IFITM1	BF210063	8519	2,19	up	11	11p15.5
Known Gene	interferon, alpha-inducible protein (clone IFI-15K)	G1P2	BM916335	9636	3,16	up	1	1p36.33
Known Gene	interferon, alpha-inducible protein 27	IFI27	BQ053892	3429	2,34	up	14	14q32
Known Gene	interferon-induced protein with tetratricopeptide repeats 1	IFIT1	AK095515	3434	4,04	up	10	10q25-q26
Known Gene	interferon-induced protein with tetratricopeptide repeats 3	IFIT3	NM_001549	3437	2,09	up	10	10q24
Known Gene	lectin, galactoside-binding, soluble, 3 binding protein	LGALS3BP	BC015761	3959	2,44	up	17	17q25
Known Gene	major histocompatibility complex, class I, B	HLA-B	BF345566	3106	2,22	up	6	6p21.3
Known Gene	myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)	MX1	AK096355	4599	5,04	up	21	21q22.3
Known Gene	myxovirus (influenza virus) resistance 2 (mouse)	MX2	AK122952	4600	2,76	up	21	21q22.3
Known Gene	nephroblastoma overexpressed gene	NOV	NM_002514	4856	2,45	up	8	8q24.1
Known Gene	polypyrimidine tract binding protein 1	PTBP1	NM_002819	5725	2,23	up	19	19p13.3
Known Gene	protein kinase C, nu	PRKCN	NM_005813	23683	2,83	up	2	2p21
Known Gene	S100 calcium binding protein, beta (neural)	S100B	BC041935	6285	3,23	up	21	21q22.3
Known Gene	solute carrier family 16 (monocarboxylic acid transporters), member 6	SLC16A6	NM_004694	9120	2,26	up	17	17q24.3
Known Gene	solute carrier family 39 (zinc transporter), member 6	SLC39A6	NM_012319	25800	2,32	up	18	18q12.2
Known Gene	solute carrier family 7 (cationic amino acid transporter, y+ system), member 8	SLC7A8	Y18483	23428	2,12	up	14	14q11.2
Known Gene	solute carrier family 7 (cationic amino acid transporter, y+ system) member 11	SLC7A11	NM_014331	23657	2,29	up	4	4q28-q32
Known Gene	sorting nexin 9	SNX9	NM_016224	51429	2,14	up	6	6q25.1-q26
Known Gene	WAP four-disulfide core domain 1	WFDC1	AK128711	58189	2,73	up	16	16q24.3
Known Gene	activating transcription factor 4 (tax-responsive enhancer element B67)	ATF4	NM_001675	468	2,04	down	22	22q13.1
Known Gene	caveolin 1, caveolae protein, 22kDa	CAV1	NM_001753	857	2,48	down	7	7q31.1
Known Gene	DNA-damage-inducible transcript 4	DDIT4	NM_019058	54541	2,41	down	10	10pter-q26.12
Known Gene	follistatin-like 1	FSTL1	BX647421	11167	2,24	down	3	3q13.33
Known Gene	growth arrest-specific 5	GAS5	AA055501	60674	2,08	down	1	1q23.3
Known Gene	growth arrest-specific 6	GAS6	AK126533	2621	2,69	down	13	13q34
Known Gene	high mobility group AT-hook 1	HMGA1	BC071863	3159	2,25	down	6	6p21
Known Gene	myosin, light polypeptide 9, regulatory	MYL9	BM701565	10398	2,33	down	20	20q11.23
Known Gene	N-myc downstream regulated gene 1	NDRG1	AK124705	10397	2,53	down	8	8q24.3
Known Gene	phosphoserine phosphatase-like	PSPHL	N75009	8781	2,47	down	7	7q11.2
Known Gene	PRO1073 protein	PRO1073	BX538238	29005	2,21	down	11	11cen-q12.3
Known Gene	proteolipid protein 1 (Pelizaeus-Merzbacher disease, spastic paraplegia 2, uncomplicated)	PLP1	NM_000533	5354	2,12	down	X	Xq22
Known Gene	thioredoxin interacting protein	TXNIP	NM_006472	10628	2,54	down	1	1q21.2
Known Gene	tumor differentially expressed 2	TDE2	AF087902	57515	2,10	down	6	6q22.32
Unknown Gene	-	-	H63873	-	2,35	up	-	-
Unknown Gene	-	-	R25643	-	2,16	up	-	-

APPENDIX S3 **Table listing full set of differentially modulated genes due to genotype x lesional type interaction**

Known / unknown	Gene name	Gene symbol	Accession number	LocusLink ID	DE	Up / down	Chromosome	Cytoband
Known Gene	24-dehydrocholesterol reductase	DHCR24	BC011669	1718	4,18	up	1	1p33-p31.1
Known Gene	5'-nucleotidase, ecto (CD73)	NT5E	BC065937	4907	15,09	up	6	6q14-q21
Known Gene	6-pyruvoyltetrahydropterin synthase	PTS	BG249563	5805	2,17	up	11	11q22.3-q23.3
Known Gene	a disintegrin and metalloproteinase domain 9 (meltrin gamma)	ADAM9	NM_003816	8754	2,99	up	8	8p11.22
Known Gene	actin, beta	ACTB	BC053988	60	2,36	up	7	7p15-p12
Known Gene	acyl-CoA synthetase long-chain family member 4	ACSL4	NM_022977	2182	2,35	up	X	Xq22.3-q23
Known Gene	ADP-ribosylation factor 4	ARF4	BC016325	378	2,86	up	3	3p21.2-p21.1
Known Gene	ATP citrate lyase	ACLY	NM_001096	47	2,00	up	17	17q12-q21
Known Gene	basic leucine zipper and W2 domains 1	BZW1	AL833518	9689	2,17	up	2	2q33
Known Gene	BCL2-like 1	BCL2L1	AY491779	598	3,29	up	20	20q11.21
Known Gene	brain expressed, X-linked 1	BEX1	BM804232	55859	4,47	up	X	Xq21-q23
Known Gene	butyrophilin-like 9	BTNL9	NM_152547	153579	2,62	up	5	5q35.3
Known Gene	bystin-like	BYSL	AK095253	705	2,33	up	6	6p22.1
Known Gene	calmodulin 2 (phosphorylase kinase, delta)	CALM2	BC047553	805	3,45	up	2	2p21
Known Gene	calponin 2	CNN2	NM_004368	1265	2,56	up	19	21q11.1
Known Gene	casein kinase 1, delta	CSNK1D	AK128863	1453	2,21	up	17	17q25
Known Gene	caspase 1, apoptosis-related cysteine protease (interleukin 1, beta, convertase)	CASP1	NM_033292	834	2,42	up	11	11q23
Known Gene	caveolin 1, caveolae protein, 22kDa	CAV1	NM_001753	857	6,41	up	7	7q31.1
Known Gene	CD109 antigen (Gov platelet alloantigens)	CD109	NM_133493	135228	2,18	up	6	6q14.1
Known Gene	CDC28 protein kinase regulatory subunit 1B	CKS1B	BQ278454	1163	3,04	up	1	1q21.2
Known Gene	CDC28 protein kinase regulatory subunit 2	CKS2	BQ898943	1164	2,56	up	9	9q22
Known Gene	CDC-like kinase 4	CLK4	BC063116	57396	3,40	up	5	5q35
Known Gene	cell division cycle 2, G1 to S and G2 to M	CDC2	AK027271	983	2,61	up	10	10q21.1
Known Gene	centromere protein F, 350/400ka (mitosin)	CENPF	NM_016343	1063	2,44	up	1	1q32-q41
Known Gene	chromosome 13 open reading frame 6	C13orf6	NM_032859	84945	2,14	up	13	13q33.3
Known Gene	chromosome 14 open reading frame 31	C14orf31	BX648295	122786	2,80	up	14	14q22.1
Known Gene	chromosome 18 open reading frame 10	C18orf10	NM_015476	25941	2,28	up	18	18q12.2
Known Gene	coiled-coil domain containing 2	CCDC2	AK023707	80173	9,22	up	9	9p21.1
Known Gene	collagen, type I, alpha 2	COL1A2	J03464	1278	16,18	up	7	7q22.1
Known Gene	collagen, type IV, alpha 2	COL4A2	NM_001846	1284	2,14	up	13	13q34
Known Gene	cullin 4B	CUL4B	BX537641	8450	2,54	up	X	Xq23
Known Gene	cytoskeleton-associated protein 4	CKAP4	NM_006825	10970	4,22	up	12	12q24.11
Known Gene	DEAD (Asp-Glu-Ala-Asp) box polypeptide 21	DDX21	BX648405	9188	2,39	up	10	10q21
Known Gene	dimethylarginine dimethylaminohydrolase 1	DDAH1	NM_012137	23576	2,30	up	1	1p22
Known Gene	DKFZP564B167 protein	DKFZP564B167	BM926614	25874	2,28	up	1	1q24
Known Gene	egf-like module containing, mucin-like, hormone receptor-like 3	EMR3	NM_032571	84658	5,62	up	19	19p13.1
Known Gene	enolase 1, (alpha)	ENO1	AK090592	2023	6,97	up	1	1p36.3-p36.2
Known Gene	FAT tumor suppressor homolog 1 (Drosophila)	FAT	NM_005245	2195	2,85	up	4	4q34-q35
Known Gene	fatty acid desaturase 3	FADS3	AK091608	3995	2,11	up	11	11q12-q13.1
Known Gene	fer-1-like 3, myoferlin (C. elegans)	FER1L3	AF182316	26509	15,25	up	10	10q24
Known Gene	fibrillin 1 (Marfan syndrome)	FBN1	X63556	2200	4,46	up	15	15q21.1
Known Gene	fibronectin 1	FN1	NM_212482	2335	66,37	up	2	2q34
Known Gene	fibronectin leucine rich transmembrane protein 2	FLRT2	NM_013231	23768	71,48	up	14	14q24-q32
Known Gene	filamin B, beta (actin binding protein 278)	FLNB	AF043045	2317	2,66	up	3	3p14.3
Known Gene	FK506 binding protein 1A, 12kDa	FKBP1A	BG107659	2280	2,51	up	20	20p13
Known Gene	folate hydrolase (prostate-specific membrane antigen) 1	FOLH1	NM_004476	2346	2,97	up	11	11p11.2
Known Gene	four and a half LIM domains 2	FHL2	BC036085	2274	3,35	up	2	2q12-q14
Known Gene	FXYD domain containing ion transport regulator 5	FXYD5	BX648809	53827	2,00	up	19	19q12-q13.1
Known Gene	general transcription factor IIA, 1, 19/37kDa	GTF2A1	NM_015859	2957	2,40	up	14	14q31.1
Known Gene	glucan (1,4-alpha-), branching enzyme 1 (glycogen branching enzyme, Andersen disease, glycogen storage disease type IV)	GBE1	AK125918	2632	4,56	up	3	3p12.3
Known Gene	glucose phosphate isomerase	GPI	BX641158	2821	2,02	up	19	19q13.1
Known Gene	glutaminase	GLS	NM_014905	2744	2,07	up	2	2q32-q34
Known Gene	glutaredoxin (thioltransferase)	GLRX	AK125636	2745	3,37	up	5	5q14
Known Gene	guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide 3	GNAI3	NM_006496	2773	2,21	up	1	1p13
Known Gene	guanine nucleotide binding protein (G protein), gamma 11	GNG11	BF971151	2791	2,79	up	7	7q31-q32
Known Gene	hect domain and RLD 4	HERC4	NM_015601	26091	2,20	up	10	10q21.3
Known Gene	hematological and neurological expressed 1	HN1	BC039343	51155	2,58	up	17	17q25.2
Known Gene	hepatoma-derived growth factor (high-mobility group protein 1-like)	HDGF	NM_004494	3068	2,39	up	1	Xq25
Known Gene	hexokinase 2	HK2	NM_000189	3099	2,91	up	2	2p13
Known Gene	high mobility group AT-hook 1	HMGA1	BU739829	3159	5,16	up	6	6p21
Known Gene	high-mobility group box 2	HMGB2	BU729607	3148	2,64	up	4	4q31
Known Gene	HSPC150 protein similar to ubiquitin-conjugating enzyme	HSPC150	BF690859	29089	2,04	up	1	1q32.1
Known Gene	hypothetical protein FLJ12442	FLJ12442	AK091962	64943	2,13	up	3	3p21.31
Known Gene	hypothetical protein FLJ13213	FLJ13213	AK055195	79811	4,56	up	15	15q21.3

APPENDIX S3 (continued)

Known / unknown	Gene name	Gene symbol	Accession number	LocusLink ID	DE	Up / down	Chromosome	Cytoband
Known Gene	hypothetical protein FLJ13273	FLJ13273	BX648355	79807	2,20	up	4	4q25
Known Gene	hypothetical protein MGC4677	MGC4677	BC070200	112597	4,70	up	2	2p11.2
Known Gene	hypoxia-inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)	HIF1A	NM_001530	3091	2,90	up	14	14q21-q24
Known Gene	immediate early response 3	IER3	BM994398	8870	2,09	up	6	6p21.3
Known Gene	inhibin, beta A (activin A, activin AB alpha polypeptide)	INHBA	BX648811	3624	3,70	up	7	7p15-p13
Known Gene	insulin-like growth factor binding protein 2, 36kDa	IGFBP2	BQ894155	3485	3,47	up	2	2q33-q34
Known Gene	insulin-like growth factor binding protein 3	IGFBP3	BX640961	3486	5,08	up	7	7p13-p12
Known Gene	integrin, alpha 3 (antigen CD49C, alpha 3 subunit of VLA-3 receptor)	ITGA3	M59911	3675	2,74	up	17	17q21.33
Known Gene	interleukin 24	IL24	NM_006850	11009	2,66	up	1	1q32
Known Gene	karyopherin alpha 2 (RAG cohort 1, importin alpha 1)	KPNA2	AK024833	3838	2,60	up	17	17q23.1-q23.3
Known Gene	KDEL (Lys-Asp-Glu-Leu) endoplasmic reticulum protein retention receptor 3	KDELR3	BC045747	11015	2,09	up	22	22q13.1
Known Gene	KIAA0276 protein	KIAA0276	BX537944	23142	3,02	up	4	4q12
Known Gene	KIAA1181 protein	KIAA1181	AL136753	57222	2,45	up	5	5q35.2
Known Gene	killer cell lectin-like receptor subfamily B, member 1	KLRB1	BC027885	3820	2,34	up	12	12p13
Known Gene	lactate dehydrogenase A	LDHA	BM994401	3939	7,76	up	11	11p15.4
Known Gene	lamin B2	LMNB2	NM_032737	84823	2,13	up	19	19p13.3
Known Gene	laminin, gamma 1 (formerly LAMB2)	LAMC1	NM_002293	3915	2,68	up	1	1q31
Known Gene	layilin	LOC143903	AK096925	143903	2,09	up	11	11q23.2
Known Gene	lectin, galactoside-binding, soluble, 1 (galectin 1)	LGALS1	AK124370	3956	2,78	up	22	22q13.1
Known Gene	LIM and SH3 protein 1	LASP1	NM_006148	3927	2,18	up	17	17q11-q21.3
Known Gene	low density lipoprotein receptor (familial hypercholesterolemia)	LDLR	BX648281	3949	3,02	up	19	19p13.3
Known Gene	membrane metallo-endopeptidase (neutral endopeptidase, enkephalinase, CALLA, CD10)	MME	NM_007289	4311	11,82	up	3	3q25.1-q25.2
Known Gene	metallothionein 1G	MT1G	BG612801	4495	2,58	up	16	16q13
Known Gene	metallothionein 1H	MT1H	BF674156	4496	2,24	up	16	16q13
Known Gene	metallothionein 1X	MT1X	BM458221	4501	2,61	up	16	16q13
Known Gene	metallothionein 2A	MT2A	BF131637	4502	2,08	up	16	16q13
Known Gene	microsomal glutathione S-transferase 1	MGST1	AK123482	4257	6,36	up	12	12p12.3-p12.1
Known Gene	microtubule-associated protein 1B	MAP1B	NM_005909	4131	4,62	up	5	5q13
Known Gene	moesin	MSN	BC017293	4478	2,25	up	X	Xq11.2-q12
Known Gene	myelin protein zero-like 1	MPZL1	NM_003953	9019	2,80	up	1	1q23.3
Known Gene	myosin regulatory light chain MRCL3	MRCL3	AK057715	10627	2,49	up	18	18p11.31
Known Gene	myosin, light polypeptide 6, alkali, smooth muscle and non-muscle	MYL6	BG292067	4637	3,33	up	12	12q13.13
Known Gene	myosin, light polypeptide 9, regulatory	MYL9	BM701565	10398	3,40	up	20	20q11.23
Known Gene	NAD(P)H dehydrogenase, quinone 1	NQO1	NM_000903	1728	4,29	up	16	16q22.1
Known Gene	nidogen (enactin)	NID	BC045606	4811	2,09	up	1	1q43
Known Gene	non-metastatic cells 1, protein (NM23A) expressed in	NME1	BM809638	4830	3,12	up	17	17q21.3
Known Gene	nuclear RNase III Drosha	RNASE3L	NM_013235	29102	2,21	up	5	5p13.3
Known Gene	nucleoside phosphorylase	NP	AK126154	4860	2,03	up	14	14q13.1
Known Gene	peptidylglycine alpha-amidating monooxygenase	PAM	NM_000919	5066	4,66	up	5	5q14-q21
Known Gene	peroxiredoxin 1	PRDX1	BF185567	5052	2,15	up	1	1p34.1
Known Gene	phosphoglycerate kinase 1	PGK1	NM_000291	5230	2,96	up	X	Xq13
Known Gene	pituitary tumor-transforming 1	PTTG1	BQ278502	9232	3,59	up	5	5q35.1
Known Gene	polymerase (RNA) II (DNA directed) polypeptide L, 7.6kDa	POLR2L	BM919305	5441	3,89	up	11	11p15
Known Gene	polymerase I and transcript release factor	PTRF	NM_012232	284119	3,72	up	17	17q21.31
Known Gene	procollagen-lysine, 2-oxoglutarate 5-dioxygenase (lysine hydroxylase) 2	PLOD2	NM_182943	5352	7,69	up	3	3q23-q24
Known Gene	procollagen-lysine, 2-oxoglutarate 5-dioxygenase (lysine hydroxylase, Ehlers-Danlos syndrome type VI)	PLOD	NM_000302	5351	2,68	up	1	1p36.3-p36.2
Known Gene	procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), alpha polypeptide I	P4HA1	BX648829	5033	3,69	up	10	10q21.3-q23.1
Known Gene	protease, serine, 11 (IGF binding)	PRSS11	BC031082	5654	3,69	up	10	10q25.3
Known Gene	protein arginine N-methyltransferase 3	PRMT3	AK125039	10196	2,53	up	11	11p15.1
Known Gene	protein phosphatase 1, regulatory (inhibitor) subunit 3C	PPP1R3C	BX537399	5507	2,69	up	10	10q23-q24
Known Gene	protein regulator of cytokinesis 1	PRC1	NM_003981	9055	3,16	up	15	15q26.1
Known Gene	proteolipid protein 2 (colonic epithelium-enriched)	PLP2	BF214130	5355	2,19	up	X	Xp11.23
Known Gene	putative small membrane protein NID67	NID67	AK125286	85027	4,31	up	5	5q33.1
Known Gene	pyruvate kinase, muscle	PKM2	NM_182470	5315	3,11	up	15	15q22
Known Gene	RAB23, member RAS oncogene family	RAB23	NM_016277	51715	2,19	up	6	6p11
Known Gene	RAB31, member RAS oncogene family	RAB31	NM_006868	11031	2,09	up	18	18p11.3
Known Gene	receptor-interacting serine-threonine kinase 2	RIPK2	AY358814	8767	2,19	up	8	8q21
Known Gene	related RAS viral (r-ras) oncogene homolog 2	RRAS2	BQ228116	22800	2,02	up	11	11p15.2
Known Gene	renal tumor antigen	RAGE	AK131542	5891	2,01	up	14	14q32
Known Gene	reticulocalbin 1, EF-hand calcium binding domain	RCN1	AK126419	5954	4,01	up	11	11p13
Known Gene	reticulon 4	RTN4	NM_020532	57142	2,88	up	2	2p16.3
Known Gene	S100 calcium binding protein A16	S100A16	NM_080388	140576	5,00	up	1	1q21
Known Gene	scavenger receptor cysteine-rich type 1 protein M160	M160	NM_174941	283316	3,87	up	12	12p13.31
Known Gene	SEC13-like 1 (S. cerevisiae)	SEC13L1	AK095629	6396	14,53	up	3	3p25-p24

APPENDIX S3 (continued)

Known / unknown	Gene name	Gene symbol	Accession number	LocusLink ID	DE	Up / down	Chromosome	Cytoband
Known Gene	SEC24 related gene family, member D (S. cerevisiae)	SEC24D	BC035761	9871	2,10	up	4	4q27
Known Gene	secreted frizzled-related protein 1	SFRP1	BC036503	6422	11,88	up	8	8p12-p11.1
Known Gene	secreted protein, acidic, cysteine-rich (osteonectin)	SPARC	AK126525	6678	2,52	up	5	5q31.3-q32
Known Gene	septin 11	SEPT 11	AK001711	55752	5,19	up	4	4q2*.1
Known Gene	serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 7	SERPINB7	BX641156	8710	7,27	up	18	18q22.1
Known Gene	serine (or cysteine) proteinase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium derived factor), member 1	SERPINF1	BM918904	5176	2,87	up	17	17p13.1
Known Gene	serine (or cysteine) proteinase inhibitor, clade H (heat shock protein 47), member 1, (collagen binding protein 1)	SERPINH1	AK122936	871	3,16	up	11	11q13.5
Known Gene	serine/threonine kinase 6	STK6	NM_198433	6790	2,12	up	20	20q13.2-q13.3
Known Gene	SET and MYND domain containing 3	SMYD3	AK023594	64754	3,32	up	1	1q44
Known Gene	Sjogren syndrome antigen B (autoantigen La)	SSB	AK124811	6741	2,68	up	2	2q3*.1
Known Gene	Smith-Magenis syndrome chromosome region, candidate 7	SMCR7	BC051466	125170	2,45	up	17	17p11.2
Known Gene	solute carrier family 1 (neutral amino acid transporter), member 5	SLC1A5	U53347	6510	4,79	up	19	19q13.3
Known Gene	solute carrier family 20 (phosphate transporter), member 1	SLC20A1	NM_005415	6574	4,49	up	2	2q11-q14
Known Gene	solute carrier family 38, member 2	SLC38A2	NM_018976	54407	9,35	up	12	12q
Known Gene	solute carrier family 39 (zinc transporter), member 10	SLC39A10	AB033091	57181	2,42	up	2	2q32.3
Known Gene	solute carrier family 7 (cationic amino acid transporter, y+ system) member 11	SLC7A11	NM_014331	23657	3,58	up	4	4q28-q32
Known Gene	spermatid perinuclear RNA binding protein	STRBP	NM_018387	55342	3,61	up	9	9q33.3
Known Gene	spondin 2, extracellular matrix protein	SPON2	AK024499	10417	2,73	up	4	4p16.3
Known Gene	squalene epoxidase	SQLE	BX647605	6713	2,10	up	8	8q24.1
Known Gene	sterol-C4-methyl oxidase-like	SC4MOL	BC010653	6307	2,49	up	4	4q32-q34
Known Gene	sulfotransferase family, cytosolic, 1C, member 1	SULT1C1	NM_176825	6819	2,26	up	2	2q11.1-q11.2
Known Gene	sushi-repeat-containing protein, X-linked 2	SRPX2	NM_014467	27286	2,11	up	X	Xq21.33-q23
Known Gene	TCDD-inducible poly(ADP-ribose) polymerase	TIPARP	BC050350	25976	3,03	up	3	3q25.31
Known Gene	thymidylate synthetase	TYMS	BQ056428	7298	3,13	up	18	18p1*.32
Known Gene	thymosin, beta 4, X-linked	TMSB4X	BM994376	7114	15,85	up	X	Xq21.3-q22
Known Gene	tissue inhibitor of metalloproteinase 3 (Sorsby fundus dystrophy, pseudoinflammatory)	TIMP3	NM_000362	7078	2,39	up	22	22q12.1-q13.2
Known Gene	transforming growth factor, beta-induced, 68kDa	TGFBI	BC026352	7045	5,33	up	5	5q31
Known Gene	translocation associated membrane protein 2	TRAM2	D31762	9697	7,29	up	6	6p21.1-p12
Known Gene	transmembrane 4 superfamily member 9	TM4SF9	AK126702	10098	3,27	up	4	4q23
Known Gene	transmembrane, prostate androgen induced RNA	TMEPAI	NM_020182	56937	3,02	up	20	20q13.31-q13.33
Known Gene	triosephosphate isomerase 1	TPI1	BM913099	7167	2,82	up	12	12p13
Known Gene	tropomyosin 1 (alpha)	TPM1	BX648171	7168	2,22	up	15	15q22.1
Known Gene	tubulin, alpha 1 (testis specific)	TUBA1	AK054731	7277	2,00	up	2	2q36.1
Known Gene	tubulin, beta 6	TUBB6	NM_032525	84617	3,07	up	18	18p1*.21
Known Gene	ubiquitin carboxyl-terminal esterase L1 (ubiquitin thiolesterase)	UCHL1	AK055249	7345	2,09	up	4	4p14
Known Gene	ubiquitin-conjugating enzyme E2Q (putative)	UBE2Q	NM_017582	55585	2,48	up	1	1q22
Known Gene	UDP-Gal:betaGlcNAc beta 1,4- galactosyltransferase, polypeptide 1	B4GALT1	NM_001497	2683	4,63	up	9	9p13
Known Gene	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 1 (GalNAc-T1)	GALNT1	NM_020474	2589	3,21	up	18	18q12.1
Known Gene	uridine monophosphate kinase	UMPK	BX640859	7371	2,03	up	1	1q23
Known Gene	vacuolar protein sorting 13D (yeast)	VPS13D	BC051804	55187	2,46	up	1	1p36.22-p36.21
Known Gene	vascular endothelial growth factor C	VEGFC	NM_005429	7424	2,12	up	4	4q34.1-q34.3
Known Gene	vinculin	VCL	BX537994	7414	2,68	up	10	10q22.1-q23
Known Gene	vitamin D (1,25- dihydroxyvitamin D3) receptor	VDR	NM_000376	7421	2,18	up	12	12q12-q14
Known Gene	WAP four-disulfide core domain 1	WFDC1	AK128711	58189	3,19	up	16	16q24.3
Known Gene	wingless-type MMTV integration site family, member 5A	WNT5A	NM_003392	7474	3,65	up	3	3p21-p14
Known Gene	zinc finger protein 606	ZNF606	BC038370	57507	3,09	up	5	5q23.2
Known Gene	ZW10 interactor	ZWINT	AK093808	11130	2,06	up	10	10q21-q22
Known Gene	2',3'-cyclic nucleotide 3' phosphodiesterase	CNP	NM_033133	1267	3,01	down	17	17q21
Known Gene	2',5'-oligoadenylate synthetase 1, 40/46kDa	OAS1	AK123528	4938	4,14	down	12	12q24.1
Known Gene	3-hydroxyisobutyrate dehydrogenase	HIBADH	AK025558	11112	2,23	down	7	7p15.2
Known Gene	6-pyruvoyl-tetrahydropterin synthase/dimerization cofactor of hepatocyte nuclear factor 1 alpha (TCF1)	PCBD	BM550965	5092	2,46	down	10	10q22
Known Gene	a disintegrin and metalloproteinase domain 10	ADAM10	NM_001110	102	2,46	down	15	15q2
Known Gene	active BCR-related gene	ABR	AK124547	29	2,72	down	17	17p13.3
Known Gene	adducin 1 (alpha)	ADD1	BC042998	118	2,36	down	4	4p16.3
Known Gene	adiponectin receptor 1	ADIPOR1	AK124455	51094	2,15	down	1	1p36.13-q41
Known Gene	ADP-ribosyltransferase (NAD+; poly (ADP-ribose) polymerase)	ADPRT	NM_001618	142	2,32	down	1	1q41-q42
Known Gene	amyotrophic lateral sclerosis 2 (juvenile) chromosome region, candidate 3	ALS2CR3	NM_015049	66008	2,27	down	2	2q33
Known Gene	angiopoietin-like 2	ANGPTL2	NM_012098	23452	4,16	down	9	9q34
Known Gene	anterior pharynx defective 1B-like	PSFL	BM563192	83464	2,18	down	15	15q22.1
Known Gene	ATPase, H+ transporting, lysosomal V0 subunit a isoform 1	ATP6V0A1	AK125927	535	3,01	down	17	17q21
Known Gene	basic helix-loop-helix domain containing, class B, 3	BHLHB3	AB044088	79365	4,68	down	12	12p11.23-p12.1
Known Gene	BET1 homolog (S. cerevisiae)	BET1	NM_005868	10282	2,00	down	7	7q21.1-q22
Known Gene	beta-site APP-cleaving enzyme 2	BACE2	NM_012105	25825	3,61	down	21	21q22.3
Known Gene	biliverdin reductase A	BLVRA	BX647539	644	3,04	down	7	7p14-cen

APPENDIX S3 (continued)

Known / unknown	Gene name	Gene symbol	Accession number	LocusLink ID	DE	Up / down	Chromosome	Cytoband
Known Gene	BMP and activin membrane-bound inhibitor homolog (Xenopus laevis)	BAMBI	NM_012342	25805	3,33	down	10	10p12.3-p11.2
Known Gene	brain expressed X-linked-like 1	BEXL1	XM_043653	56271	2,15	down	X	Xq22.1-q22.3
Known Gene	brevican	BCAN	NM_021948	63827	2,94	down	1	1q31
Known Gene	bridging integrator 3	BIN3	AK090446	55909	2,30	down	8	8p21.2
Known Gene	butyrophilin, subfamily 3, member A3	BTN3A3	NM_006994	10384	2,94	down	6	6p21.3
Known Gene	cadherin 1, type 1, E-cadherin (epithelial)	CDH1	NM_004360	999	7,03	down	16	16q22.1
Known Gene	cadherin 3, type 1, P-cadherin (placental)	CDH3	BC041846	1001	3,14	down	16	16q22.1
Known Gene	calreticulin	CALR	M84739	811	2,56	down	19	19p13.3-p13.2
Known Gene	cAMP responsive element binding protein-like 2	CREB_2	AF039081	1389	2,64	down	12	12p13
Known Gene	carbonic anhydrase XIV	CA14	BM701119	23632	4,26	down	1	1q21
Known Gene	cathepsin F	CTSF	BC013359	8722	4,03	down	11	11q13
Known Gene	cathepsin H	CTSH	AK026152	1512	2,23	down	15	15q24-q25
Known Gene	cathepsin K (pycnodysostosis)	CTSK	NM_000396	1513	4,32	down	1	1q21
Known Gene	CCAAT/enhancer binding protein zeta	CEBPZ	NM_005760	10153	2,16	down	2	2p22.2
Known Gene	CD9 antigen (p24)	CD9	BM709541	928	2,26	down	12	12p13.3
Known Gene	CDC-like kinase 2	CLK2	AK091036	1196	2,25	down	1	1q21
Known Gene	cell division cycle 2-like 5 (cholinesterase-related cell division controller)	CDC2L5	NM_003718	8621	3,28	down	7	7p13
Known Gene	centaurin, gamma 1	CENTG1	D79989	116986	2,29	down	12	12q13.2
Known Gene	ceroid-lipofuscinosis, neuronal 2, late infantile (Jansky-Bielschowsky disease)	CLN2	NM_000391	1200	4,83	down	11	11p15
Known Gene	CGI-51 protein	CGI-51	AK124895	25813	2,64	down	22	22q13.31
Known Gene	chemokine (C-C motif) receptor 7	CCR7	NM_001838	1236	2,35	down	17	17q12-q21.2
Known Gene	chemokine (C-X3-C motif) receptor 1	CX3CR1	BC028078	1524	2,08	down	3	3p21
Known Gene	chloride channel 7	CLCN7	AK122944	1186	2,49	down	16	16p13
Known Gene	chromosome 1 open reading frame 38	C1orf38	AK094833	9473	3,34	down	1	1p35.3
Known Gene	chromosome 11 open reading frame 10	C11orf10	CD049126	746	2,36	down	11	11q12-q13.1
Known Gene	chromosome 2 open reading frame 24	C2orf24	NM_015680	27013	3,06	down	2	2q35
Known Gene	chromosome 20 open reading frame 26	C20orf26	NM_015585	26074	9,12	down	20	20p11.23
Known Gene	chromosome 20 open reading frame 43	C20orf43	BM703857	51507	2,14	down	20	20q13.31
Known Gene	chromosome 20 open reading frame 98	C20orf98	AL136915	80023	3,17	down	20	20p13
Known Gene	chromosome 5 open reading frame 5	C5orf5	AF251038	51306	5,04	down	5	5q31
Known Gene	chromosome 6 open reading frame 48	C6orf48	BQ057480	50854	3,24	down	6	6p21.3
Known Gene	c-mer proto-oncogene tyrosine kinase	MERTK	NM_006343	10461	2,10	down	2	2q14.1
Known Gene	crystallin, alpha B	CRYAB	BU734674	1410	4,81	down	11	11q22.3-q23.1
Known Gene	crystallin, lambda 1	CRYL1	BC071810	51084	2,51	down	13	13q12.11
Known Gene	CUG triplet repeat, RNA binding protein 1	CUGBP1	NM_198700	10658	2,65	down	11	11p11
Known Gene	CUG triplet repeat, RNA binding protein 2	CUGBP2	AF090693	10659	3,69	down	10	10p13
Known Gene	cyclin-dependent kinase inhibitor 1B (p27, Kip1)	CDKN1B	NM_004064	1027	2,39	down	12	12p13.1-p12
Known Gene	cystatin B (stefin B)	CSTB	BG121809	1476	3,17	down	21	21q22.3
Known Gene	cytochrome P450, family 3, subfamily A, polypeptide 4	CYP3A4	NM_017460	1576	3,63	down	7	7q21.1
Known Gene	DEAD (Asp-Glu-Ala-Asp) box polypeptide 48	DDX48	BM478434	9775	2,25	down	17	17q25.3
Known Gene	delta sleep inducing peptide, immunoreactor	DSIPI	NM_198057	1831	2,24	down	X	Xq22.3
Known Gene	diacylglycerol kinase, zeta 104kDa	DGKZ	U94905	8525	2,29	down	11	11p11.2
Known Gene	dipeptidylpeptidase 4 (CD26, adenosine deaminase complexing protein 2)	DPP4	NM_001935	1803	4,96	down	2	2q24.3
Known Gene	DnaJ (Hsp40) homolog, subfamily C, member 13	DNAJC13	AY369172	23317	2,29	down	3	3q22.1
Known Gene	DNase II-like acid DNase	DLAD	NM_021233	58511	5,09	down	1	1p22.3
Known Gene	dopachrome tautomerase (dopachrome delta-isomerase, tyrosine-related protein 2)	DCT	AJ000503	1638	22,42	down	13	13q32
Known Gene	dual specificity phosphatase 3 (vaccinia virus phosphatase VH1-related)	DUSP3	NM_004090	1845	2,71	down	17	17q21
Known Gene	ectonucleotide pyrophosphatase/phosphodiesterase 2 (autotaxin)	ENPP2	AK124910	5168	3,85	down	8	8q24.1
Known Gene	endothelin receptor type B	EDNRB	NM_000115	1910	2,05	down	13	13q22
Known Gene	epidermal growth factor receptor pathway substrate 15	EPS15	NM_001981	2060	3,77	down	1	1p32
Known Gene	epoxide hycrolase 1, microsomal (xenobiotic)	EPHX1	NM_000120	2052	4,26	down	1	1q42.1
Known Gene	eukaryotic translation initiation factor 4E binding protein 3	EIF4EBP3	AF445025	8637	2,10	down	5	5q31.3
Known Gene	fasciculation and elongation protein zeta 1 (zygin I)	FEZ1	NM_022549	9638	2,45	down	11	11q24.2
Known Gene	F-box protein 7	FBXO7	BX648151	25793	3,07	down	22	22q12-q13
Known Gene	FERM, RhoGEF (ARHGEF) and pleckstrin domain protein 1 (chondrocyte-derivec)	FARP1	BX537667	10160	2,03	down	13	13q32.2-q32.3
Known Gene	FERM, RhoGEF and pleckstrin domain protein 2	FARP2	AB018336	9855	3,07	down	2	2q37.3
Known Gene	FK506 binding protein 5	FKBP5	BC042605	2289	2,37	down	6	6p21.3-21.2
Known Gene	FK506 binding protein 7	FKBP7	NM_016105	51661	2,63	down	2	2q31.3
Known Gene	G protein-coupled receptor 143	GPR143	Z48804	4935	2,58	down	X	Xp22.3
Known Gene	G protein-coupled receptor 158	GPR158	NM_020752	57512	3,89	down	10	10p12.1
Known Gene	G protein-coupled receptor 56	GPR56	NM_201524	9289	5,50	down	16	16q13
Known Gene	GABA(A) receptor-associated protein	GABARAP	BM803698	11337	2,08	down	17	17p13.2
Known Gene	GABA(A) receptor-associated protein like 1	GABARAPL1	BX648781	23710	2,18	down	12	12p13.31
Known Gene	galactose-3-O-sulfotransferase 4	GAL3ST4	NM_024637	79690	3,07	down	7	7q22

APPENDIX S3 (continued)

Known / unknown	Gene name	Gene symbol	Accession number	LocusLink ID	DE	Up / down	Chromosome	Cytoband
Known Gene	general transcription factor IIA, 1, 19/37kDa	GTF2A1	BC037828	2957	2,07	down	14	14q31.1
Known Gene	glial fibrillary acidic protein	GFAP	BC062609	2670	2,12	down	17	17q21
Known Gene	glioblastoma amplified sequence	GBAS	AK125036	2631	2,66	down	7	7p12
Known Gene	glucosamine-6-phosphate deaminase 1	GNPDA1	NM_005471	10007	2,10	down	5	5q21
Known Gene	glucuronidase, beta	GUSB	AK096764	2990	3,55	down	7	7q21.11
Known Gene	glutathione S-transferase kappa 1	GSTK1	NM_015917	373156	2,13	down	7	-
Known Gene	glycogenin 2	GYG2	NM_003918	8908	3,09	down	X	Xp22.3
Known Gene	glycoprotein (transmembrane) nmb	GPNMB	BC032783	10457	9,17	down	7	7p15
Known Gene	GM2 ganglioside activator	GM2A	XM_379364	2760	3,46	down	5	5q31.3-q33.1
Known Gene	GRIP and coiled-coil domain containing 1	GCC1	BC014100	79571	2,01	down	7	7q32.2
Known Gene	growth differentiation factor 15	GDF15	AK025948	9518	3,31	down	19	19p13.1-13.2
Known Gene	growth hormone receptor	GHR	NM_000163	2690	2,13	down	5	5p13-p12
Known Gene	guanosine monophosphate reductase	GMPR	BM994423	2766	3,10	down	6	6p23
Known Gene	guanylate kinase 1	GUK1	AK124677	2987	2,76	down	1	1q32-q41
Known Gene	heat shock 22kDa protein 8	HSPB8	NM_014365	26353	3,04	down	12	12q24.23
Known Gene	heme binding protein 1	HEBP1	AK131565	50865	2,25	down	12	12p13.2
Known Gene	Hermansky-Pudlak syndrome 4	HPS4	NM_152840	89781	3,05	down	22	22cen-q12.3
Known Gene	histamine N-methyltransferase	HNMT	NM_006895	3176	2,03	down	2	2q22.1
Known Gene	HLA complex group 4	HCG4	AF036977	54435	2,89	down	6	6p21.3
Known Gene	HMG-box transcription factor 1	HBP1	NM_012257	26959	2,28	down	7	7q22-q31
Known Gene	HP1-BP74	HP1-BP74	BC053327	50809	2,73	down	1	1p36.12
Known Gene	HSPC244	MGC:13379	NM_016499	51259	3,61	down	11	11q13.1
Known Gene	hydroxyprostaglandin dehydrogenase 15-(NAD)	HPGD	NM_000860	3248	2,68	down	4	4q34-q35
Known Gene	hypothetical protein Ells1	Ells1	AL834391	222166	7,54	down	7	7p15.1
Known Gene	hypothetical protein FLJ10157	FLJ10157	XM_371354	55083	4,28	down	1	1q44
Known Gene	hypothetical protein FLJ10357	FLJ10357	XM_370737	55701	2,27	down	14	14q11.2
Known Gene	hypothetical protein FLJ11193	FLJ11193	BC032845	55322	2,03	down	5	5p13.3
Known Gene	hypothetical protein FLJ11767	FLJ11767	NM_024593	79645	4,82	down	8	8q11.21
Known Gene	hypothetical protein FLJ14525	FLJ14525	BC066649	84886	4,33	down	1	1q42.13-q43
Known Gene	hypothetical protein FLJ20303	FLJ20303	AK055456	54888	2,05	down	5	5p15.32
Known Gene	hypothetical protein FLJ20344	FLJ20344	AK097159	55634	2,23	down	X	Xp11.3
Known Gene	hypothetical protein FLJ22353	FLJ22353	AL832539	79639	2,31	down	1	1p34.1
Known Gene	hypothetical protein FLJ23441	FLJ23441	AK056980	79731	2,42	down	11	11q13.4
Known Gene	hypothetical protein MGC14156	MGC14156	BC007876	84992	2,63	down	4	4q22.1
Known Gene	hypothetical protein MGC4268	MGC4268	BC016181	83607	2,55	down	2	2q21
Known Gene	immunoglobulin (CD79A) binding protein 1	IGBP1	AK054596	3476	2,78	down	X	Xq13.1-q13.3
Known Gene	immunoglobulin superfamily, member 11	IGSF11	AK122611	152404	2,05	down	3	3q13.33
Known Gene	immunoglobulin superfamily, member 3	IGSF3	AB007935	3321	2,20	down	1	1p13
Known Gene	inositol 1,4,5-trisphosphate 3-kinase B	ITPKB	AJ242780	3707	3,86	down	1	1q42.13
Known Gene	interferon, gamma-inducible protein 16	IFI16	AF208043	3428	2,15	down	1	1q22
Known Gene	interferon-induced protein with tetratricopeptide repeats 1	IFIT1	AK095515	3434	3,17	down	10	10q25-q26
Known Gene	interferon-stimulated transcription factor 3, gamma 48kDa	ISGF3G	BC035716	10379	2,07	down	14	14q11.2
Known Gene	JM1 protein	JM1	BC000972	28952	4,19	down	X	Xp11.23
Known Gene	KIAA0087 gene product	KIAA0087	XM_379798	9808	3,68	down	7	7p15.2
Known Gene	KIAA0367	KIAA0367	XM_041018	23273	3,25	down	9	9q21.31
Known Gene	KIAA0830 protein	KIAA0830	BC071171	23052	2,09	down	11	11q21
Known Gene	KIAA0830 protein	KIAA0830	XM_290546	23052	4,65	down	11	11q21
Known Gene	KIAA0934 protein	KIAA0934	AB023151	22982	3,32	down	10	10p15.3
Known Gene	KIAA1026 protein	KIAA1026	AB028949	23254	2,30	down	1	1p36.13
Known Gene	KIAA1450 protein	KIAA1450	AB040883	57600	2,00	down	4	4q32.1
Known Gene	KIAA1958	KIAA1958	AL390067	158405	2,32	down	9	9q32
Known Gene	lectin, galactoside-binding, soluble, 3 (galectin 3)	LGALS3	AK091776	3958	2,39	down	14	14q21-q22
Known Gene	lectin, galactoside-binding, soluble, 3 binding protein	LGALS3BP	BC015761	3959	4,40	down	17	17q25
Known Gene	likely ortholog of mouse monocyte macrophage 19	MMRF19	AF131812	51074	2,47	down	11	11p13
Known Gene	lipopolysaccharide-induced TNF factor	LITAF	AB034747	9516	2,54	down	16	16p13.3-p12
Known Gene	lysosomal-associated membrane protein 2	LAMP2	BX648255	3920	2,12	down	X	Xq24
Known Gene	macrophage migration inhibitory factor (glycosylation-inhibiting factor)	MIF	BQ056329	4282	8,07	down	22	22q11.23
Known Gene	MAD2 mitotic arrest deficient-like 2 (yeast)	MAD2L2	AK094316	10459	2,28	down	1	1p36
Known Gene	major histocompatibility complex, class I, A	HLA-A	AK125608	3105	4,54	down	6	6p21.3
Known Gene	major histocompatibility complex, class I, B	HLA-B	BF345569	3106	3,21	down	6	6p21.3
Known Gene	major histocompatibility complex, class I, C	HLA-C	AK127349	3107	3,00	down	6	6p21.3
Known Gene	major histocompatibility complex, class I, E	HLA-E	X56841	3133	2,96	down	6	6p21.3
Known Gene	makorin, ring finger protein, 1	MKRN1	AK127030	23608	2,17	down	7	7q34
Known Gene	mannosidase, beta A, lysosomal-like	MANBAL	AK026708	63905	2,70	down	20	20q11.23-q12

APPENDIX S3 (continued)

Known / unknown	Gene name	Gene symbol	Accession number	LocusLink ID	DE	Up / down	Chromosome	Cytoband
Known Gene	MARCKS-like protein	MLP	BU168218	65108	2,67	down	1	1p34.3
Known Gene	MAX interactor 1	MXI1	NM_130439	4601	2,57	down	10	10q24-q25
Known Gene	MCF.2 cell line derived transforming sequence	MCF2	AL117234	4168	2,77	down	X	Xq27
Known Gene	melan-A	MLANA	BF573723	2315	4,65	down	9	9p24.1
Known Gene	melanophilin	MLPH	AK096789	79083	3,13	down	2	2q37.3
Known Gene	metastasis suppressor 1	MTSS1	AB007889	9788	2,51	down	8	8p22
Known Gene	MGC4170 protein	MGC4170	NM_024312	79158	2,51	down	12	12q23.3
Known Gene	microphthalmia-associated transcription factor	MITF	NM_198159	4286	4,22	down	3	3p14.2-p14.1
Known Gene	microtubule-associated protein, RP/EB family, member 1	MAPRE1	BC040410	22919	2,15	down	20	20q11.1-11.23
Known Gene	mitochondrial ribosomal protein L44	MRPL44	AK095007	65080	3,90	down	2	2q36.3
Known Gene	MYC-associated zinc finger protein (purine-binding transcription factor)	MAZ	AK097472	4150	2,54	down	16	16p11.2
Known Gene	myelin expression factor 2	MYEF2	AB037762	50804	2,73	down	15	15q15.2
Known Gene	myosin ID	MYO1D	AB018270	4642	4,26	down	17	17q11-q12
Known Gene	MYST histone acetyltransferase 1	MYST1	BC037773	84148	5,05	down	16	16p11.2
Known Gene	myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)	MX1	AK096355	4599	3,81	down	21	21q22.3
Known Gene	myxovirus (influenza virus) resistance 2 (mouse)	MX2	AK122952	4600	2,53	down	21	21q22.3
Known Gene	NAD(P)H:quinone oxidoreductase type 3, polypeptide A2	NQO3A2		51706	2,95	down	1	1p36.13-q41
Known Gene	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 5, 13kDa	NDUFA5	BF576548	4698	2,17	down	7	7q32
Known Gene	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 5, 16kDa	NDUFB5	BX648034	4711	2,00	down	3	3q27.1
Known Gene	NEDD9 interacting protein with calponin homology and LIM domains	NICAL	AK024500	64780	3,92	down	6	6q21
Known Gene	neural precursor cell expressed, developmentally down-regulated 4-like	NEDD4L	AB007899	23327	3,53	down	18	18q21
Known Gene	neuroblastoma, suppression of tumorigenicity 1	NBL1	NM_182744	4681	5,05	down	1	1p36.13-p36.11
Known Gene	neuronal pentraxin I	NPTX1	AK127335	4884	2,02	down	17	17q25.1-q25.2
Known Gene	nuclear factor I/B	NFIB	BX537698	4781	2,29	down	9	9p24.1
Known Gene	nuclear receptor coactivator 5	NCOA5	BC056872	57727	4,04	down	20	20q12-c13.12
Known Gene	nucleoporin 93kDa	NUP93	NM_014669	9688	2,03	down	16	16q13
Known Gene	oculocutaneous albinism II (pink-eye dilution homolog, mouse)	OCA2	NM_000275	4948	4,29	down	15	15q11.2-q12
Known Gene	olfactomedin 1	OLFM1	NM_014279	10439	2,97	down	9	9q34.3
Known Gene	oxysterol binding protein-like 9	OSBP_9	AK124037	114883	2,69	down	1	1p32.3
Known Gene	peptidase D	PEPD	BC004305	5184	2,11	down	19	19q12-q13.2
Known Gene	peripheral myelin protein 22	PMP22	NM_000304	5376	4,15	down	17	17p12-p11.2
Known Gene	phosphodiesterase 4D interacting protein (myomegalin)	PDE4DIP	AL833273	9659	2,24	down	1	1q12
Known Gene	phosphoinositol 3-phosphate-binding protein-2	PEPP2	AB051473	54477	3,14	down	12	12p12
Known Gene	phospholipid transfer protein	PLTP	AK097009	5360	2,17	down	20	20q12-q13.1
Known Gene	phosphoprotein associated with glycosphingolipid-enriched microdomains	PAG	AK000680	55824	2,79	down	8	8q21.12
Known Gene	phosphoserine phosphatase-like	PSPHL	BC065228	8781	3,62	down	7	7q11.2
Known Gene	pirin (iron-binding nuclear protein)	PIR	BX537579	8544	4,73	down	X	Xp22.31
Known Gene	plastin 3 (T isoform)	PLS3	BC056898	5358	2,10	down	X	Xq24
Known Gene	plexin C1	PLXNC1	AF030339	10154	4,34	down	12	12q23.3
Known Gene	poly(A) binding protein, cytoplasmic 4 (inducible form)	PABPC4	AK125926	8761	2,47	down	1	1p32-p36
Known Gene	polymerase (RNA) II (DNA directed) polypeptide F	POLR2F	AL832562	5435	3,72	down	22	22q13.1
Known Gene	potassium voltage-gated channel, shaker-related subfamily, beta member 2	KCNAB2	AK124696	8514	2,48	down	1	1p36.3
Known Gene	prosaposin (variant Gaucher disease and variant metachromatic leukodystrophy)	PSAP	BC007612	5660	3,22	down	10	10q21-q22
Known Gene	prostatic binding protein	PBP	BM921938	5037	2,32	down	12	12q24.23
Known Gene	protein phosphatase 1H (PP2C domain containing)	PPM1H	BC040177	57460	2,29	down	12	12q14.1-q14.2
Known Gene	protein tyrosine phosphatase type IVA, member 1	PTP4A1	NM_003463	7803	2,96	down	6	6q12
Known Gene	protein tyrosine phosphatase, non-receptor type substrate 1	PTPNS1	BC038510	140885	2,66	down	20	20p13
Known Gene	protein tyrosine phosphatase, receptor type, f polypeptide (PTPRF), interacting protein (liprin), alpha 1	PPFIA1	NM_003626	8500	2,17	down	11	11q13.2
Known Gene	proteolipid protein 1 (Pelizaeus-Merzbacher disease, spastic paraplegia 2, uncomplicated)	PLP1	NM_000533	5354	4,75	down	X	Xq22
Known Gene	PTPRF interacting protein, binding protein 2 (liprin beta 2)	PPFIBP2	BC021714	8495	3,06	down	11	11p15.4
Known Gene	putative nuclear protein ORF1-FL49	ORF1-FL49	BM462754	84418	2,96	down	5	5q31.3
Known Gene	RAB38, member RAS oncogene family	RAB38	BC015808	23682	3,42	down	11	11q14
Known Gene	RAB5B, member RAS oncogene family	RAB5B	BX537408	5869	2,42	down	12	12q13
Known Gene	ras homolog gene family, member C	RHOC	BC009177	389	2,13	down	1	1p13.1
Known Gene	Ras-related GTP binding C	RRAGC	AK092808	64121	2,05	down	1	1p34
Known Gene	regulator of G-protein signalling 12	RGS12	NM_002926	6002	2,27	down	4	4p16.3
Known Gene	regulator of G-protein signalling 20	RGS20	NM_170587	8601	3,05	down	8	8q12.1
Known Gene	retinoic acid induced 17	RAI17	AY235683	57178	2,15	down	10	10q23.1
Known Gene	S100 calcium binding protein, beta (neural)	S100B	BC041935	6285	2,34	down	21	21q22.3
Known Gene	scavenger receptor class B, member 1	SCARB1	AK023485	949	2,87	down	12	12q24.31
Known Gene	SEC22 vesicle trafficking protein-like 3 (S. cerevisiae)	SEC22L3	AK074576	9117	2,23	down	3	3p21.33
Known Gene	secreted phosphoprotein 1 (osteopontin, bone sialoprotein I, early T-lymphocyte activation 1)	SPP1	BF698898	6696	9,34	down	4	4q21-q25
Known Gene	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3C	SEMA3C	NM_006379	10512	3,28	down	7	7q21-q31
Known Gene	sema domain, immunoglobulin domain (Ig), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 4C	SEMA4C	AB051526	54910	2,22	down	2	2q11.2

APPENDIX S3 (continued)

Known / unknown	Gene name	Gene symbol	Accession number	LocusLink ID	DE	Up / down	Chromosome	Cytoband
Known Gene	sema domain, transmembrane domain (TM), and cytoplasmic domain, (semaphorin) 6A	SEMA6A	BC032619	57556	2,49	down	5	5q23.1
Known Gene	serine/threonine kinase 24 (STE20 homolog, yeast)	STK24	BC035578	8428	2,61	down	13	13q31.2-q32.3
Known Gene	sialyltransferase 7 ((alpha-N-acetylneuraminyl-2,3-beta-galactosyl-1,3)-N-acetyl galactosaminide alpha-2,6-sialyltransferase) C	SIAT7C	BX648274	256435	4,58	down	1	1p31.1
Known Gene	sialyltransferase 9 (CMP-NeuAc:lactosylceramide alpha-2,3-sialyltransferase; GM3 synthase)	SIAT9	AK127346	8869	2,14	down	2	2p11.2
Known Gene	Sin3A associated protein p30-like	SAP30L	AL833381	79685	2,28	down	5	5q33.2
Known Gene	SKI interacting protein	SKIIP	NM_012245	22938	2,42	down	14	14q24.3
Known Gene	solute carrier family 1 (glutamate/neutral amino acid transporter), member 4	SLC1A4	AK126636	6509	4,24	down	2	2p15-p13
Known Gene	solute carrier family 12 (potassium/chloride transporters), member 7	SLC12A7	NM_006598	10723	2,01	down	5	5p15
Known Gene	solute carrier family 2 (facilitated glucose transporter), member 1	SLC2A1	NM_006516	6513	2,06	down	1	1p35-p31.3
Known Gene	solute carrier family 27 (fatty acid transporter), member 3	SLC27A3	AK074134	11000	2,34	down	1	1q22
Known Gene	solute carrier family 6 (neurotransmitter transporter), member 15	SLC6A15	NM_018057	55117	3,00	down	12	12q21.3
Known Gene	solute carrier family 6 (neurotransmitter transporter, creatine), member 8	SLC6A8	NM_005629	6535	2,15	down	X	Xq28
Known Gene	solute carrier family 8 (sodium/calcium exchanger), member 1	SLC8A1	N88818	6546	3,39	down	2	2p23-22
Known Gene	sorbin and SH3 domain containing 1	SORBS1	AL117472	10580	2,46	down	10	10q23.3-q24.1
Known Gene	sorting nexin 19	SNX19	XM_374949	9795	4,33	down	11	11q25
Known Gene	sparc/osteonectin, cwcv and kazal-like domains proteoglycan (testican)	SPOCK	BC030691	6695	3,05	down	5	5q31
Known Gene	spectrin, beta, non-erythrocytic 5	SPTBN5	AF233523	51332	3,55	down	15	15q21
Known Gene	spermidine/spermine N1-acetyltransferase	SAT	BF680536	6303	3,13	down	X	Xp22.1
Known Gene	Spi-B transcription factor (Spi-1/PU.1 related)	SPIB	AK025419	6689	2,31	down	19	19q13.3-q13.4
Known Gene	splicing factor 3a, subunit 3, 60kDa	SF3A3	AK128438	10946	3,80	down	1	1p34.2
Known Gene	SRY (sex determining region Y)-box 10	SOX10	BC018808	6663	3,18	down	22	22q13.1
Known Gene	SRY (sex determining region Y)-box 4	SOX4	NM_003107	6659	3,30	down	6	6p22.3
Known Gene	stannin	SNN	NM_003498	8303	2,29	down	16	16p13
Known Gene	stathmin 1/oncoprotein 18	STMN1	BX647885	3925	2,09	down	1	1p36.1-p35
Known Gene	suppressior of tumorigenicity 5	ST5	NM_005418	6764	2,14	down	11	11p15
Known Gene	suppressior of tumorigenicity 7	ST7	NM_013437	29967	3,32	down	8	8q22.2-q23.1
Known Gene	synaptojanin 2 binding protein	SYNJ2BP	AK123967	55333	2,25	down	14	14q24.1
Known Gene	syndecan binding protein (syntenin)	SDCBP	AK126645	6386	2,67	down	8	8q12
Known Gene	synuclein, alpha (non A4 component of amyloid precursor)	SNCA	L08850	6622	2,53	down	4	4q21
Known Gene	target of myb1 (chicken)	TOM1	AK097926	10043	4,37	down	22	22q13.1
Known Gene	TBC1 domain family, member 16	TBC1D16	NM_019020	125058	3,52	down	17	17q25.3
Known Gene	TBC1 domain family, member 7	TBC1D7	AL833474	51256	2,42	down	6	6p23
Known Gene	tetratricopeptide repeat domain 3	TTC3	NM_003316	7267	3,31	down	21	21q22.2
Known Gene	transducin-like enhancer of split 1 (E(sp1) homolog, Drosophila)	TLE1	NM_005077	7088	3,37	down	9	9q21.32
Known Gene	transforming, acidic coiled-coil containing protein 1	TACC1	AF049910	6867	4,25	down	8	8p11
Known Gene	transmembrane 7 superfamily member 1 (upregulated in kidney)	TM7SF1	AL832142	7107	3,10	down	1	1q42-q43
Known Gene	transmembrane protein 24	TMEM24	NM_014807	9854	3,11	down	11	11q23.3
Known Gene	tribbles homolog 2	TRB2	NM_021643	28951	4,64	down	2	2p24.3
Known Gene	tripartite motif-containing 2	TRIM2	AF220018	23321	2,38	down	4	4q31.3
Known Gene	tRNA nucleotidyl transferase, CCA-adding, 1	TRNT1	NM_182916	51095	2,04	down	3	
Known Gene	truncated calcium binding protein	LOC51149	BX647375	51149	2,57	down	5	5q35.3
Known Gene	tudor domain containing 3	TDRD3	BX537910	81550	2,34	down	13	13q21.1
Known Gene	tweety homolog 2 (Drosophila)	TTYH2	AK126955	94015	3,42	down	17	
Known Gene	tyrosinase-related protein 1	TYRP1	X51420	7306	3,95	down	9	9p23
Known Gene	ubiquitin-like 3	UBL3	BC044582	5412	2,13	down	13	13q12-q13
Known Gene	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 10 (GalNAc-T10)	GALNT10	NM_198321	55568	2,09	down	5	5q33.2
Known Gene	vacuolar protein sorting 41 (yeast)	VPS41	BX648347	27072	3,05	down	7	7p14-p13
Known Gene	vascular endothelial growth factor B	VEGFB	BM701452	7423	2,89	down	11	11q13
Known Gene	very low density lipoprotein receptor	VLDLR	NM_003383	7436	3,16	down	9	9p24
Known Gene	vesicle amine transport protein 1 homolog (T californica)	VAT1	BC015041	10493	3,48	down	17	17q21
Known Gene	v-fos FBJ murine osteosarcoma viral oncogene homolog	FOS	BX647104	2353	2,77	down	14	14q24.3
Known Gene	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	KIT	BC071593	3815	2,49	down	4	4q11-q12
Known Gene	v-maf musculoaponeurotic fibrosarcoma oncogene homolog (avian)	MAF	AF055376	4094	3,22	down	16	16q22-q23
Known Gene	WD40 repeat protein interacting with phosphoInositides of 49kDa	WIPI49	NM_024682	55062	2,82	down	17	17q24.2
Known Gene	zinc finger protein 14 (KOX 6)	ZNF14	NM_021030	7561	2,47	down	19	19p13.3-p13.2
Known Gene	zinc finger, DHHC domain containing 4	ZDHHC4	NM_018106	55146	2,68	down	7	7p22.2
Unknown Gene	-	-	H80239	-	16,49	up	-	-
Unknown Gene	-	-	AA026379	-	2,38	up	-	-
Unknown Gene	-	-	AU126203	-	2,11	up	-	-
Unknown Gene	-	-	N58330	-	3,94	up	-	-
Unknown Gene	-	-	R16197	-	2,86	up	-	-
Unknown Gene	-	-	R21740	-	2,24	up	-	-
Unknown Gene	-	-	T89619	-	2,80	up	-	-
Unknown Gene	-	-	W80366	-	2,32	up	-	-

APPENDIX S3 (continued)

Known / unknown	Gene name	Gene symbol	Accession number	LocusLink ID	DE	Up / down	Chromosome	Cytoband
Unknown Gene	-	-	N36881	-	2,10	down	-	-
Unknown Gene	-	-	R33626	-	3,72	down	-	-
Unknown Gene	-	-	R43685	-	2,99	down	-	-
Unknown Gene	-	-	AA403186	-	3,08	down	-	-
Unknown Gene	-	-	AA807681	-	4,14	down	-	-
Unknown Gene	-	-	H05313	-	2,69	down	-	-
Unknown Gene	-	-	N28925	-	3,39	down	-	-
Unknown Gene	-	-	N71258	-	4,37	down	-	-
Unknown Gene	-	-	R07879	-	2,06	down	-	-
Unknown Gene	-	-	R12947	-	3,10	down	-	-
Unknown Gene	-	-	R13855	-	4,73	down	-	-
Unknown Gene	-	-	R33139	-	3,69	down	-	-
Unknown Gene	-	-	R42887	-	2,03	down	-	-
Unknown Gene	-	-	R68102	-	2,10	down	-	-
Unknown Gene	-	-	R86658	-	3,08	down	-	-
Unknown Gene	-	-	R87728	-	2,24	down	-	-
Unknown Gene	-	-	T84043	-	2,46	down	-	-

APPENDIX S4 **Table listing the biological process categories of differentially modulated genes due to genotype effect**

GO ID	Biological process	Corrected p-value	Gene name	Gene symbol	LocusLink ID	DE	Up / down
GO:0008151	cell growth and/or maintenance	0,00074	cyclin D1 (PRAD1: parathyroid adenomatosis 1)	CCND1	595	4.00	up
GO:0008151	cell growth and/or maintenance	0,00074	v-fos FBJ murine osteosarcoma viral oncogene homolog	FOS	2353	2.21	down
GO:0008151	cell growth and/or maintenance	0,00074	follistatin-like 3 (secreted glycoprotein)	FSTL3	10272	2.42	up
GO:0008151	cell growth and/or maintenance	0,00074	immediate early response 3	IER3	8870	2.20	up
GO:0008151	cell growth and/or maintenance	0,00074	v-maf musculoaponeurotic fibrosarcoma oncogene homolog (avian)	MAF	4094	2.14	down
GO:0008151	cell growth and/or maintenance	0,00074	prohibitin	PHB	5245	2.15	up
GO:0008151	cell growth and/or maintenance	0,00074	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3C	SEMA3C	10512	2.01	down
GO:0006955	immune response	0,00304	accessory protein BAP31	BCAP31	10134	2.14	up
GO:0006955	immune response	0,00304	major histocompatibility complex, class I, E	HLA-E	3133	2.27	down
GO:0006955	immune response	0,00304	interferon, alpha-inducible protein 27	IFI27	3429	2.31	down
GO:0006955	immune response	0,00304	macrophage migration inhibitory factor (glycosylation-inhibiting factor)	MIF	4282	2.66	down
GO:0006955	immune response	0,00304	myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)	MX1	4599	5.04	down
GO:0006955	immune response	0,00304	myxovirus (influenza virus) resistance 2 (mouse)	MX2	4600	2.76	down
GO:0006955	immune response	0,00304	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3C	SEMA3C	10512	2.01	down
GO:0006886	intracellular protein transport	0,00012	adaptor-related protein complex 1, sigma 2 subunit	AP1S2	8905	2.86	down
GO:0006886	intracellular protein transport	0,00012	adaptor-related protein complex 2, sigma 1 subunit	AP2S1	1175	2.00	up
GO:0006886	intracellular protein transport	0,00012	accessory protein BAP31	BCAP31	10134	2.14	up
GO:0006886	intracellular protein transport	0,00012	coatomer protein complex, subunit epsilon	COPE	11316	2.15	up
GO:0006886	intracellular protein transport	0,00012	N-ethylmaleimide-sensitive factor attachment protein, alpha	NAPA	8775	2.00	up
GO:0006886	intracellular protein transport	0,00012	sorting nexin 17	SNX17	9784	2.03	up
GO:0008285	negative regulation of cell proliferation	0,00716	glycoprotein (transmembrane) nmb	GPNMB	10457	3.00	down
GO:0008285	negative regulation of cell proliferation	0,00716	prohibitin	PHB	5245	2.15	up
GO:0008285	negative regulation of cell proliferation	0,00716	transducer of ERBB2, 1	TOB1	10140	2.06	up
GO:0006915	apoptosis	0,04871	accessory protein BAP31	BCAP31	10134	2.14	up
GO:0006915	apoptosis	0,04871	death associated transcription factor 1	DATF1	11083	2.11	up
GO:0006915	apoptosis	0,04871	immediate early response 3	IER3	8870	2.20	up
GO:0007001	chromosome organization and biogenesis (sensu Eukarya)	0,00260	high mobility group AT-hook 1	HMGA1	3159	2.90	up
GO:0007001	chromosome organization and biogenesis (sensu Eukarya)	0,00260	SMC2 structural maintenance of chromosomes 2-like 1 (yeast)	SMC2L1	10592	2.04	up
GO:0007010	cytoskeleton organization and biogenesis	0,00281	apolipoprotein E	APOE	348	2.90	up
GO:0007010	cytoskeleton organization and biogenesis	0,00281	NEDD9 interacting protein with calponin homology and LIM domains	NICAL	64780	2.31	down
GO:0007010	cytoskeleton organization and biogenesis	0,00281	thymosin, beta 4, X-linked	TMSB4X	7114	2.11	up
GO:0006917	induction of apoptosis	0,00678	apolipoprotein E	APOE	348	2.90	up
GO:0006917	induction of apoptosis	0,00678	caspase 4, apoptosis-related cysteine protease	CASP4	837	2.06	down
GO:0006917	induction of apoptosis	0,00678	myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)	MX1	4599	5.04	down
GO:0000398	nuclear mRNA splicing, via spliceosome	0,00675	splicing factor 3a, subunit 3, 60kDa	SF3A3	10946	2.16	down
GO:0000398	nuclear mRNA splicing, via spliceosome	0,00675	splicing factor, arginine/serine-rich 1 (splicing factor 2, alternate splicing factor)	SFRS1	6426	2.15	up
GO:0000398	nuclear mRNA splicing, via spliceosome	0,00675	small nuclear ribonucleoprotein polypeptides B and B1	SNRPB	6628	3.73	up
GO:0045941	positive regulation of transcription	0,00017	high mobility group AT-hook 1	HMGA1	3159	2.90	up
GO:0045941	positive regulation of transcription	0,00017	interferon regulatory factor 4	IRF4	3662	2.01	down
GO:0006461	protein complex assembly	0,01182	high mobility group AT-hook 1	HMGA1	3159	2.90	up
GO:0006461	protein complex assembly	0,01182	transmembrane 4 superfamily member 7	TM4SF7	7106	2.03	up
GO:0007264	small GTPase mediated signal transduction	0,03647	ADP-ribosylation factor-like 7	ARL7	10123	2.11	down
GO:0006366	transcription from Pol II promoter	0,03630	v-maf musculoaponeurotic fibrosarcoma oncogene homolog (avian)	MAF	4094	2.14	down
GO:0006366	transcription from Pol II promoter	0,03630	Pirin	PIR	8544	3.02	down
GO:0006366	transcription from Pol II promoter	0,03630	polymerase (RNA) II (DNA directed) polypeptide E, 25kDa	POLR2E	5434	2.20	up
GO:0019735	antimicrobial humoral response (sensu Vertebrata)	0,02385	coatomer protein complex, subunit epsilon	COPE	11316	2.15	up
GO:0007166	cell surface receptor linked signal transduction	0,04774	HMT1 hnRNP methyltransferase-like 2 (S. cerevisiae)	HRMT1L2	3276	2.62	up
GO:0007166	cell surface receptor linked signal transduction	0,04774	natriuretic peptide receptor B/guanylate cyclase B (atrionatriuretic peptide receptor B)	NPR2	4882	2.13	up
GO:0006952	defense response	0,01306	HMT1 hnRNP methyltransferase-like 2 (S. cerevisiae)	HRMT1L2	3276	2.62	up
GO:0006952	defense response	0,01306	myeloid leukemia factor 2	MLF2	8079	2.01	up
GO:0006268	DNA unwinding	0,00025	high mobility group AT-hook 1	HMGA1	3159	2.90	up
GO:0006096	glycolysis	0,00742	aldolase A, fructose-bisphosphate	ALDOA	226	2.22	up
GO:0006096	glycolysis	0,00742	enolase 1, (alpha)	ENO1	2023	2.38	up
GO:0007156	homophilic cell adhesion	0,01864	cadherin 1, type 1, E-cadherin (epithelial)	CDH1	999	2.04	down
GO:0006629	lipid metabolism	0,04844	ceroid-lipofuscinosis, neuronal 2, late infantile (Jansky-Bielschowsky disease)	CLN2	1200	2.05	down
GO:0006629	lipid metabolism	0,04844	phospholipase A1 member A	PLA1A	51365	3.12	down
GO:0006345	loss of chromatin silencing	0,00001	high mobility group AT-hook 1	HMGA1	3159	2.90	up
GO:0007067	mitosis	0,02314	MAD2 mitotic arrest deficient-like 2 (yeast)	MAD2L2	10459	2.26	down
GO:0007067	mitosis	0,02314	SMC2 structural maintenance of chromosomes 2-like 1 (yeast)	SMC2L1	10592	2.04	up
GO:0009653	morphogenesis	0,03325	immediate early response 3	IER3	8870	2.20	up
GO:0009653	morphogenesis	0,03325	SRY (sex determining region Y)-box 10	SOX10	6663	2.27	down
GO:0007517	muscle development	0,03720	myosin, light polypeptide 9, regulatory	MYL9	10398	3.50	up
GO:0007517	muscle development	0,03720	transgelin 2	TAGLN2	8407	3.62	up
GO:0006337	nucleosome disassembly	0,00004	high mobility group AT-hook 1	HMGA1	3159	2.90	up

APPENDIX S4 (continued)

GO ID	Biological process	Corrected p-value	Gene name	Gene symbol	LocusLink ID	DE	Up / down
GO:0006813	potassium ion transport	0,04865	ATPase, Na+/K+ transporting, alpha 1 polypeptide	ATP1A1	476	2.09	up
GO:0006813	potassium ion transport	0,04865	potassium voltage-gated channel, shaker-related subfamily, beta member 2	KCNAB2	8514	2.34	down
GO:0006464	protein modification	0,02087	beta-site APP-cleaving enzyme 2	BACE2	25825	2.42	down
GO:0006464	protein modification	0,02087	peptidylglycine alpha-amidating monooxygenase	PAM	5066	3.74	down
GO:0009306	protein secretion	0,00249	beta-site APP-cleaving enzyme 2	BACE2	25825	2.42	down
GO:0009306	protein secretion	0,00249	protein transport protein SEC61 alpha subunit isoform 1	SEC61A1	29927	2.59	up
GO:0030100	regulation of endocytosis	0,00001	adaptor-related protein complex 2, sigma 1 subunit	AP2S1	1175	2.00	up
GO:0030100	regulation of endocytosis	0,00001	sorting nexin 17	SNX17	9784	2.03	up
GO:0008277	regulation of G-protein coupled receptor protein signaling pathway	0,00368	regulator of G-protein signalling 12	RGS12	6002	2.02	up
GO:0008277	regulation of G-protein coupled receptor protein signaling pathway	0,00368	regulator of G-protein signalling 20	RGS20	8601	2.23	down
GO:0042493	response to drug	0,00075	major vault protein	MVP	9961	2.26	up
GO:0042493	response to drug	0,00075	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3C	SEMA3C	10512	2.01	down
GO:0008380	RNA splicing	0,01632	splicing factor 3a, subunit 3, 60kDa	SF3A3	10946	2.16	down
GO:0008380	RNA splicing	0,01632	small nuclear ribonucleoprotein polypeptides B and B1	SNRPB	6628	3.73	up
GO:0007271	synaptic transmission cholinergic	0,00008	apolipoprotein E	APOE	348	2.90	up
GO:0007271	synaptic transmission cholinergic	0,00008	NAD(P)H dehydrogenase, quinone 1	NQO1	1728	2.40	down
GO:0006350	transcription	0,02239	death associated transcription factor 1	DATF1	11083	2.11	up
GO:0006350	transcription	0,02239	polymerase (RNA) II (DNA directed) polypeptide E, 25kDa	POLR2E	5434	2.20	up
GO:0030036	actin cytoskeleton organization and biogenesis	0,03287	syncecan binding protein (syntenin)	SDCBP	6386	2.03	down
GO:0019883	antigen presentation, endogenous antigen	0,00474	major histocompatibility complex, class I, E	HLA-E	3133	2.27	down
GO:0019885	antigen processing, endogenous antigen via MHC class I	0,00857	major histocompatibility complex, class I, E	HLA-E	3133	2.27	down
GO:0015991	ATP hydrolysis coupled proton transport	0,00125	ATPase, Na+/K+ transporting, alpha 1 polypeptide	ATP1A1	476	2.09	up
GO:0016051	carbohydrate biosynthesis	0,00459	glycogenin 2	GYG2	8908	2.01	down
GO:0006182	cGMP biosynthesis	0,00580	natriuretic peptide receptor B/guanylate cyclase B (atrionatriuretic peptide receptor B)	NPR2	4882	2.13	up
GO:0006695	cholesterol biosynthesis	0,01306	24-dehydrocholesterol reductase	DHCR24	1718	2.17	up
GO:0042632	cholesterol homeostasis	0,00000	apolipoprotein E	APOE	348	2.90	up
GO:0007059	chromosome segregation	0,01185	SMC2 structural maintenance of chromosomes 2-like 1 (yeast)	SMC2L1	10592	2.04	up
GO:0008015	circulation	0,03708	apolipoprotein E	APOE	348	2.90	up
GO:0048268	clathrin cage assembly	0,00000	adaptor-related protein complex 2, sigma 1 subunit	AP2S1	1175	2.00	up
GO:0030866	cortical actin cytoskeleton organization and biogenesis	0,00187	erythrocyte membrane protein band 4.1-like 3	EPB41L3	23136	2.20	up
GO:0000910	cytokinesis	0,01172	protein regulator of cytokinesis 1	PRC1	9055	2.01	up
GO:0007016	cytoskeletal anchoring	0,00652	plectin 1, intermediate filament binding protein 500kDa	PLEC1	5339	2.02	up
GO:0006835	dicarboxylic acid transport	0,00563	solute carrier family 1 (glutamate/neutral amino acid transporter), member 4	SLC1A4	6509	2.14	down
GO:0006259	DNA metabolism	0,01942	prohibitin	PHB	5245	2.15	up
GO:0006306	DNA methylation	0,00759	v-fos FBJ murine osteosarcoma viral oncogene homolog	FOS	2353	2.21	down
GO:0016197	endosome transport	0,00445	sorting nexin 17	SNX17	9784	2.03	up
GO:0007173	epidermal growth factor receptor signaling pathway	0,00769	epidermal growth factor receptor pathway substrate 15	EPS15	2060	2.04	down
GO:0008544	epidermis development	0,04787	dopachrome tautomerase (dopachrome delta-isomerase, tyrosine-related protein 2)	DCT	1638	3.00	down
GO:0006325	establishment and/or maintenance of chromatin architecture	0,00740	high-mobility group 20B	HMG20B	10362	2.07	up
GO:0006726	eye pigment biosynthesis	0,00121	oculocutaneous albinism II (pink-eye dilution homolog, mouse)	OCA2	4948	2.35	down
GO:0006631	fatty acid metabolism	0,04652	lysophospholipase II	LYPLA2	11313	2.28	up
GO:0006000	fructose metabolism	0,00268	aldolase A, fructose-bisphosphate	ALDOA	226	2.22	up
GO:0006004	fucose metabolism	0,00216	A kinase (PRKA) anchor protein (yotiao) 9	AKAP9	10142	2.33	up
GO:0000082	G1/S transition of mitotic cell cycle	0,01187	cyclin D1 (PRAD1: parathyroid adenomatosis 1)	CCND1	595	4.00	up
GO:0005978	glycogen biosynthesis	0,00539	glycogenin 2	GYG2	8908	2.01	down
GO:0019377	glycolipid catabolism	0,00000	GM2 ganglioside activator protein	GM2A	2760	2.05	down
GO:0006687	glycosphingolipid metabolism	0,00176	GM2 ganglioside activator protein	GM2A	2760	2.05	down
GO:0040007	growth	0,00867	sialyltransferase 1 (beta-galactoside alpha-2,6-sialyltransferase)	SIAT1	6480	2.14	down
GO:0015012	heparan sulfate proteoglycan biosynthesis	0,00000	N-deacetylase/N-sulfotransferase (heparan glucosaminyl) 1	NDST1	3340	2.09	up
GO:0016575	histone deacetylation	0,00555	prohibitin	PHB	5245	2.15	up
GO:0006959	humoral immune response	0,02217	sialyltransferase 1 (beta-galactoside alpha-2,6-sialyltransferase)	SIAT1	6480	2.14	down
GO:0030641	hydrogen ion homeostasis	0,00118	ATPase, Na+/K+ transporting, alpha 1 polypeptide	ATP1A1	476	2.09	up
GO:0007229	integrin-mediated signaling pathway	0,03570	integrin, alpha 3 (antigen CD49C, alpha 3 subunit of VLA-3 receptor)	ITGA3	3675	2.47	up
GO:0046907	intracellular transport	0,00179	apolipoprotein E	APOE	348	2.90	up
GO:0006891	intra-Golgi transport	0,00750	N-ethylmaleimide-sensitive factor attachment protein, alpha	NAPA	8775	2.00	up
GO:0007611	learning and/or memory	0,00452	apolipoprotein E	APOE	348	2.90	up
GO:0006869	lipid transport	0,04285	apolipoprotein E	APOE	348	2.90	up
GO:0042157	lipoprotein metabolism	0,00661	apolipoprotein E	APOE	348	2.90	up
GO:0045192	low-density lipoprotein catabolism	0,00053	sorting nexin 17	SNX17	9784	2.03	up
GO:0006583	melanin biosynthesis from tyrosine	0,00352	dopachrome tautomerase (dopachrome delta-isomerase, tyrosine-related protein 2)	DCT	1638	3.00	down
GO:0006944	membrane fusion	0,00877	N-ethylmaleimide-sensitive factor attachment protein, alpha	NAPA	8775	2.00	up
GO:0006509	membrane protein ectodomain proteolysis	0,00206	beta-site APP-cleaving enzyme 2	BACE2	25825	2.42	down
GO:0007076	mitotic chromosome condensation	0,00263	SMC2 structural maintenance of chromosomes 2-like 1 (yeast)	SMC2L1	10592	2.04	up
GO:0000070	mitotic sister chromatid segregation	0,00359	SMC2 structural maintenance of chromosomes 2-like 1 (yeast)	SMC2L1	10592	2.04	up

APPENDIX S4 (continued)

GO ID	Biological process	Corrected p-value	Gene name	Gene symbol	LocusLink ID	DE	Up / down
GO:0007094	mitotic spindle checkpoint	0,00211	MAD2 mitotic arrest deficient-like 2 (yeast)	MAD2L2	10459	2.26	down
GO:0000022	mitotic spindle elongation	0,00051	protein regulator of cytokinesis 1	PRC1	9055	2.01	up
GO:0030224	monocyte differentiation	0,00000	interferon, gamma-inducible protein 16	IFI16	3428	2.51	down
GO:0006397	mRNA processing	0,04633	small nuclear ribonucleoprotein polypeptides B and B1	SNRPB	6628	3.73	up
GO:0006376	mRNA splice site selection	0,00222	splicing factor, arginine/serine-rich 1 (splicing factor 2, alternate splicing factor)	SFRS1	6426	2.15	up
GO:0042985	negative regulation of amyloid precursor protein biosynthesis	0,00000	beta-site APP-cleaving enzyme 2	BACE2	25825	2.42	down
GO:0030308	negative regulation of cell growth	0,01749	enolase 1, (alpha)	ENO1	2023	2.38	up
GO:0016481	negative regulation of transcription	0,02066	prohibitin	PHB	5245	2.15	up
GO:0000122	negative regulation of transcription from Pol II promoter	0,04880	enolase 1, (alpha)	ENO1	2023	2.38	up
GO:0015804	neutral amino acid transport	0,00172	solute carrier family 1 (glutamate/neutral amino acid transporter), member 4	SLC1A4	6509	2.14	down
GO:0006809	nitric oxide biosynthesis	0,00670	NAD(P)H dehydrogenase, quinone 1	NQO1	1728	2.40	down
GO:0009117	nucleotide metabolism	0,01608	guanosine monophosphate reductase	GMPR	2766	2.75	down
GO:0009311	oligosaccharide metabolism	0,00254	sialyltransferase 1 (beta-galactoside alpha-2,6-sialyltransferase)	SIAT1	6480	2.14	down
GO:0006730	one-carbon compound metabolism	0,02204	carbonic anhydrase XIV	CA14	23632	2.13	down
GO:0001519	peptide amidation	0,00000	peptidylglycine alpha-amidating monooxygenase	PAM	5066	3.74	down
GO:0016486	peptide hormone processing	0,00133	beta-site APP-cleaving enzyme 2	BACE2	25825	2.42	down
GO:0006518	peptide metabolism	0,00115	peptidylglycine alpha-amidating monooxygenase	PAM	5066	3.74	down
GO:0006658	phosphatidylserine metabolism	0,00000	phospholipase A1 member A	PLA1A	51365	3.12	down
GO:0045082	positive regulation of interleukin-10 biosynthesis	0,00000	interferon regulatory factor 4	IRF4	3662	2.01	down
GO:0045368	positive regulation of interleukin-13 biosynthesis	0,00000	interferon regulatory factor 4	IRF4	3662	2.01	down
GO:0045086	positive regulation of interleukin-2 biosynthesis	0,00201	interferon regulatory factor 4	IRF4	3662	2.01	down
GO:0045404	positive regulation of interleukin-4 biosynthesis	0,00057	interferon regulatory factor 4	IRF4	3662	2.01	down
GO:0006476	protein amino acid deacetylation	0,00059	N-deacetylase/N-sulfotransferase (heparan glucosaminyl) 1	NDST1	3340	2.09	up
GO:0006486	protein amino acid glycosylation	0,04421	sialyltransferase 1 (beta-galactoside alpha-2,6-sialyltransferase)	SIAT1	6480	2.14	down
GO:0006479	protein amino acid methylation	0,00196	HMT1 hnRNP methyltransferase-like 2 (S. cerevisiae)	HRMT1L2	3276	2.62	up
GO:0006477	protein amino acid sulfation	0,00258	N-deacetylase/N-sulfotransferase (heparan glucosaminyl) 1	NDST1	3340	2.09	up
GO:0016579	protein deubiquitination	0,00068	ubiquitin carboxyl-terminal esterase L1 (ubiquitin thiolesterase)	UCHL1	7345	2.19	down
GO:0007243	protein kinase cascade	0,03416	Ribosomal protein s6 kinase, 90kd, polypeptide 2	RPS6KA2	6196	2.81	up
GO:0006612	protein-membrane targeting	0,00183	syncecan binding protein (syntenin)	SDCBP	6386	2.03	down
GO:0042981	regulation of apoptosis	0,04440	caspase 4, apoptosis-related cysteine protease	CASP4	837	2.06	down
GO:0030516	regulation of axon extension	0,00000	apolipoprotein E	APOE	348	2.90	up
GO:0008217	regulation of blood pressure	0,02079	natriuretic peptide receptor B/guanylate cyclase B (atrionatriuretic peptide receptor B)	NPR2	4882	2.13	up
GO:0006937	regulation of muscle contraction	0,00888	myosin, light polypeptide 9, regulatory	MYL9	10398	3.50	up
GO:0048168	regulation of neuronal synaptic plasticity	0,00062	apolipoprotein E	APOE	348	2.90	up
GO:0045622	regulation of T-helper cell differentiation	0,00000	interferon regulatory factor 4	IRF4	3662	2.01	down
GO:0006446	regulation of translational initiation	0,01819	eukaryotic translation initiation factor 4 gamma, 1	EIF4G1	1981	3.82	up
GO:0009409	response to cold	0,00129	guanosine monophosphate reductase	GMPR	2766	2.75	down
GO:0009613	response to pest/pathogen/parasite	0,01489	interferon, alpha-inducible protein 27	IFI27	3429	2.31	down
GO:0000302	response to reactive oxygen species	0,00049	apolipoprotein E	APOE	348	2.90	up
GO:0009636	response to toxin	0,00572	NAD(P)H dehydrogenase, quinone 1	NQO1	1728	2.40	down
GO:0009615	response to virus	0,01954	interferon, gamma-inducible protein 16	IFI16	3428	2.51	down
GO:0006368	RNA elongation from Pol II promoter	0,00466	general transcription factor IIF, polypeptide 2, 30kDa	GTF2F2	2963	2.00	down
GO:0030317	sperm motility	0,00547	ATPase, Na+/K+ transporting, alpha 1 polypeptide	ATP1A1	476	2.09	up
GO:0030149	sphingolipid catabolism	0,00065	GM2 ganglioside activator protein	GM2A	2760	2.05	down
GO:0006941	striated muscle contraction	0,01316	aldolase A, fructose-bisphosphate	ALDOA	226	2.22	up
GO:0006930	substrate-bound cell migration, cell extension	0,00192	syncecan binding protein (syntenin)	SDCBP	6386	2.03	down
GO:0006801	superoxide metabolism	0,01328	cytochrome b-245, alpha polypeptide	CYBA	1535	2.49	up
GO:0042110	T-cell activation	0,01505	interferon regulatory factor 4	IRF4	3662	2.01	down
GO:0006367	transcription initiation from Pol II promoter	0,03130	general transcription factor IIF, polypeptide 2, 30kDa	GTF2F2	2963	2.00	down
GO:0006805	xenobiotic metabolism	0,02744	NAD(P)H dehydrogenase, quinone 1	NQO1	1728	2.40	down

APPENDIX S5 **Table listing the biological process categories of differentially modulated genes due to lesional type effect**

GO ID	Function Name	Corrected P-Value	Gene name	Gene symbol	LocusLink ID	DE	Up / down
GO:0006955	immune response	0,00000	interferon, alpha-inducible protein (clone IFI-15K)	G1P2	9636	3.16	up
GO:0006955	immune response	0,00000	major histocompatibility complex, class I, B	HLA-B	3106	2.22	up
GO:0006955	immune response	0,00000	interferon, alpha-inducible protein 27	IFI27	3429	2.34	up
GO:0006955	immune response	0,00000	interferon-induced protein with tetratricopeptide repeats 1	IFIT1	3434	4.04	up
GO:0006955	immune response	0,00000	interferon induced transmembrane protein 1 (9-27)	IFITM1	8519	2.19	up
GO:0006955	immune response	0,00000	myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)	MX1	4599	5.04	up
GO:0006955	immune response	0,00000	myxovirus (influenza virus) resistance 2 (mouse)	MX2	4600	2.76	up
GO:0006955	immune response	0,00000	2',5'-oligoadenylate synthetase 1, 40/46kDa	OAS1	4938	3.13	up
GO:0006955	immune response	0,00000	2'-5'-oligoadenylate synthetase 2, 69/71kDa	OAS2	4939	5.25	up
GO:0006955	immune response	0,00000	2'-5'-oligoadenylate synthetase 3, 100kDa	OAS3	4940	2.44	up
GO:0006461	protein complex assembly	0,00005	high mobility group AT-hook 1	HMGA1	3159	2.25	down
GO:0006461	protein complex assembly	0,00005	solute carrier family 7, (cationic amino acid transporter, y+ system) member 11	SLC7A11	23657	2.29	up
GO:0006461	protein complex assembly	0,00005	solute carrier family 7 (cationic amino acid transporter, y+ system), member 8	SLC7A8	23428	2.12	up
GO:0007165	signal transduction	0,03467	caspase 1, apoptosis-related cysteine protease (interleukin 1, beta, convertase)	CASP1	834	2.42	up
GO:0007165	signal transduction	0,03467	growth arrest-specific 6	GAS6	2621	2.69	down
GO:0007165	signal transduction	0,03467	lectin, galactoside-binding, soluble, 3 binding protein	LGALS3BP	3959	2.44	up
GO:0007165	signal transduction	0,03467	myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)	MX1	4599	5.04	up
GO:0006139	"nucleobase, nucleoside, nucleotide and nucleic acid metabolism"	0,00004	2',5'-oligoadenylate synthetase 1, 40/46kDa	OAS1	4938	3.13	up
GO:0006139	"nucleobase, nucleoside, nucleotide and nucleic acid metabolism"	0,00004	2'-5'-oligoadenylate synthetase 2, 69/71kDa	OAS2	4939	5.25	up
GO:0006139	"nucleobase, nucleoside, nucleotide and nucleic acid metabolism"	0,00004	2'-5'-oligoadenylate synthetase 3, 100kDa	OAS3	4940	2.44	up
GO:0008283	cell proliferation	0,00498	bone marrow stromal cell antigen 2	BST2	684	4.70	up
GO:0008283	cell proliferation	0,00498	growth arrest-specific 6	GAS6	2621	2.69	down
GO:0008283	cell proliferation	0,00498	S100 calcium binding protein, beta (neural)	S100B	6285	3.23	up
GO:0006810	transport	0,02044	solute carrier family 16 (monocarboxylic acid transporters), member 6	SLC16A6	9120	2.26	up
GO:0006810	transport	0,02044	solute carrier family 7, (cationic amino acid transporter, y+ system) member 11	SLC7A11	23657	2.29	up
GO:0006810	transport	0,02044	solute carrier family 7 (cationic amino acid transporter, y+ system), member 8	SLC7A8	23428	2.12	up
GO:0006865	amino acid transport	0,00093	solute carrier family 7, (cationic amino acid transporter, y+ system) member 11	SLC7A11	23657	2.29	up
GO:0006865	amino acid transport	0,00093	solute carrier family 7 (cationic amino acid transporter, y+ system), member 8	SLC7A8	23428	2.12	up
GO:0007155	cell adhesion	0,02921	growth arrest-specific 6	GAS6	2621	2.69	down
GO:0007155	cell adhesion	0,02921	lectin, galactoside-binding, soluble, 3 binding protein	LGALS3BP	3959	2.44	up
GO:0007267	cell-cell signaling	0,01593	bone marrow stromal cell antigen 2	BST2	684	4.70	up
GO:0007267	cell-cell signaling	0,01593	interferon, alpha-inducible protein (clone IFI-15K)	G1P2	9636	3.16	up
GO:0007001	chromosome organization and biogenesis (sensu Eukarya)	0,00092	high mobility group AT-hook 1	HMGA1	3159	2.25	down
GO:0006268	DNA unwinding	0,00001	high mobility group AT-hook 1	HMGA1	3159	2.25	down
GO:0006917	induction of apoptosis	0,00218	myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)	MX1	4599	5.04	up
GO:0006917	induction of apoptosis	0,00218	S100 calcium binding protein, beta (neural)	S100B	6285	3.23	up
GO:0007242	intracellular signaling cascade	0,02650	protein kinase C, nu	PRKCN	23683	2.83	up
GO:0007242	intracellular signaling cascade	0,02650	sorting nexin 9	SNX9	51429	2.14	up
GO:0006345	loss of chromatin silencing	0,00000	high mobility group AT-hook 1	HMGA1	3159	2.25	down
GO:0006337	nucleosome disassembly	0,00000	high mobility group AT-hook 1	HMGA1	3159	2.25	down
GO:0043123	positive regulation of I-kappaB kinase/NF-kappaB cascade	0,00281	bone marrow stromal cell antigen 2	BST2	684	4.70	up
GO:0043123	positive regulation of I-kappaB kinase/NF-kappaB cascade	0,00281	caspase 1, apoptosis-related cysteine protease (interleukin 1, beta, convertase)	CASP1	834	2.42	up
GO:0045941	positive regulation of transcription	0,00010	high mobility group AT-hook 1	HMGA1	3159	2.25	down
GO:0001558	regulation of cell growth	0,00096	growth arrest-specific 6	GAS6	2621	2.69	down
GO:0001558	regulation of cell growth	0,00096	nephroblastoma overexpressed gene	NOV	4856	2.45	up
GO:0019883	"antigen presentation, endogenous antigen"	0,00055	major histocompatibility complex, class I, B	HLA-B	3106	2.22	up
GO:0019885	"antigen processing, endogenous antigen via MHC class I"	0,00106	major histocompatibility complex, class I, B	HLA-B	3106	2.22	up
GO:0000398	"nuclear mRNA splicing, via spliceosome"	0,01060	polypyrimidine tract binding protein 1	PTBP1	5725	2.23	up
GO:0006520	amino acid metabolism	0,00717	solute carrier family 7 (cationic amino acid transporter, y+ system), member 8	SLC7A8	23428	2.12	up
GO:0048143	astrocyte activation	0,00000	S100 calcium binding protein, beta (neural)	S100B	6285	3.23	up
GO:0007409	axonogenesis	0,00109	S100 calcium binding protein, beta (neural)	S100B	6285	3.23	up
GO:0006874	calcium ion homeostasis	0,00275	S100 calcium binding protein, beta (neural)	S100B	6285	3.23	up
GO:0030154	cell differentiation	0,01040	N-myc downstream regulated gene 1	NDRG1	10397	2.53	down
GO:0007166	cell surface receptor linked signal transduction	0,02009	interferon induced transmembrane protein 1 (9-27)	IFITM1	8519	2.19	up
GO:0006968	cellular defense response	0,00738	lectin, galactoside-binding, soluble, 3 binding protein	LGALS3BP	3959	2.44	up
GO:0007417	central nervous system development	0,00618	S100 calcium binding protein, beta (neural)	S100B	6285	3.23	up
GO:0006878	copper ion homeostasis	0,00022	ATX1 antioxidant protein 1 homolog (yeast)	ATOX1	475	2.05	up
GO:0006825	copper ion transport	0,00031	ATX1 antioxidant protein 1 homolog (yeast)	ATOX1	475	2.05	up
GO:0006259	DNA metabolism	0,00376	DNase II-like acid DNase	DLAD	58511	5.09	up
GO:0006112	energy reserve metabolism	0,00034	S100 calcium binding protein, beta (neural)	S100B	6285	3.23	up
GO:0006959	humoral immune response	0,00305	bone marrow stromal cell antigen 2	BST2	684	4.70	up
GO:0048151	hyperphosphorylation	0,00000	S100 calcium binding protein, beta (neural)	S100B	6285	3.23	up
GO:0006811	ion transport	0,02607	ATX1 antioxidant protein 1 homolog (yeast)	ATOX1	475	2.05	up
GO:0007611	learning and/or memory	0,00053	S100 calcium binding protein, beta (neural)	S100B	6285	3.23	up

APPENDIX S5 (continued)

GO ID	Function Name	Corrected P-Value	Gene name	Gene symbol	LocusLink ID	DE	Up / down
GO:0006869	lipid transport	0,00625	hypothetical protein DKFZp434F0318	DKFZP434F0318	81575	2.10	up
GO:0042157	lipoprotein metabolism	0,00084	hypothetical protein DKFZp434F0318	DKFZP434F0318	81575	2.10	up
GO:0006509	membrane protein ectodomain proteolysis	0,00023	beta-site APP-cleaving enzyme 2	BACE2	25825	2.02	up
GO:0030001	metal ion transport	0,00266	ATX1 antioxidant protein 1 homolog (yeast)	ATOX1	475	2.05	up
GO:0015718	monocarboxylic acid transport	0,00033	solute carrier family 16 (monocarboxylic acid transporters), member 6	SLC16A6	9120	2.26	up
GO:0007517	muscle development	0,01652	myosin, light polypeptide 9, regulatory	MYL9	10398	2.33	down
GO:0042985	negative regulation of amyloid precursor protein biosynthesis	0,00000	beta-site APP-cleaving enzyme 2	BACE2	25825	2.02	up
GO:0030308	negative regulation of cell growth	0,00291	WAP four-disulfide core domain 1	WFDC1	58189	3.19	up
GO:0008285	negative regulation of cell proliferation	0,02050	interferon induced transmembrane protein 1 (9-27)	IFITM1	8519	2.19	up
GO:0016486	peptide hormone processing	0,00012	beta-site APP-cleaving enzyme 2	BACE2	25825	2.02	up
GO:0045917	positive regulation of complement activation	0,00000	S100 calcium binding protein, beta (neural)	S100B	6285	3.23	up
GO:0007205	protein kinase C activation	0,00282	protein kinase C, nu	PRKCN	23683	2.83	up
GO:0008104	protein localization	0,00043	sorting nexin 9	SNX9	51429	2.14	up
GO:0006464	protein modification	0,01050	beta-site APP-cleaving enzyme 2	BACE2	25825	2.02	up
GO:0009306	protein secretion	0,00269	beta-site APP-cleaving enzyme 2	BACE2	25825	2.02	up
GO:0015031	protein transport	0,03061	sorting nexin 9	SNX9	51429	2.14	up
GO:0042981	regulation of apoptosis	0,00639	caspase 1, apoptosis-related cysteine protease (interleukin 1, beta, convertase)	CASP1	834	2.42	up
GO:0000074	regulation of cell cycle	0,03753	interferon induced transmembrane protein 1 (9-27)	IFITM1	8519	2.19	up
GO:0042035	regulation of cytokine biosynthesis	0,00000	S100 calcium binding protein, beta (neural)	S100B	6285	3.23	up
GO:0048169	regulation of long-term neuronal synaptic plasticity	0,00000	S100 calcium binding protein, beta (neural)	S100B	6285	3.23	up
GO:0006937	regulation of muscle contraction	0,00112	myosin, light polypeptide 9, regulatory	MYL9	10398	2.33	down
GO:0009607	response to biotic stimulus	0,00044	interferon induced transmembrane protein 1 (9-27)	IFITM1	8519	2.19	up
GO:0010038	response to metal ion	0,00041	N-myc downstream regulated gene 1	NDRG1	10397	2.53	down
GO:0006979	response to oxidative stress	0,00430	ATX1 antioxidant protein 1 homolog (yeast)	ATOX1	475	2.05	up
GO:0009613	response to pest/pathogen/parasite	0,00194	interferon, alpha-inducible protein 27	IFI27	3429	2.34	up
GO:0009615	response to virus	0,00367	2',5'-oligoadenylate synthetase 1, 40/46kDa	OAS1	4938	3.13	up
GO:0008380	RNA splicing	0,00842	polypyrimidine tract binding protein 1	PTBP1	5725	2.23	up

APPENDIX S6 **Table listing the biological process categories of differentially modulated genes due to genotype x skin lesional type interaction**

GO ID	Function Name	Corrected P-Value	Gene name	Gene symbol	LocusLink ID	DE	Up / down
GO:0007155	cell adhesion	0,00413	chondroitin sulfate proteoglycan BEHAB/brevican	BCAN	63827	2.95	down
GO:0007155	cell adhesion	0,00413	bystin-like	BYSL	705	2.33	up
GO:0007155	cell adhesion	0,00413	chromosome 1 open reading frame 38	C1orf38	9473	3.34	down
GO:0007155	cell adhesion	0,00413	CD9 antigen (p24)	CD9	928	2.26	down
GO:0007155	cell adhesion	0,00413	cadherin 3, type 1, P-cadherin (placental)	CDH3	1001	3.14	down
GO:0007155	cell adhesion	0,00413	chemokine (C-X3-C motif) receptor 1	CX3CR1	1524	2.08	down
GO:0007155	cell adhesion	0,00413	FAT tumor suppressor homolog 1 (Drosophila)	FAT	2195	2.85	up
GO:0007155	cell adhesion	0,00413	fasciculation and elongation protein zeta 1 (zygin I)	FEZ1	9638	2.45	down
GO:0007155	cell adhesion	0,00413	fibronectin leucine rich transmembrane protein 2	FLRT2	23768	71.48	up
GO:0007155	cell adhesion	0,00413	fibronectin 1	FN1	2335	69.37	up
GO:0007155	cell adhesion	0,00413	laminin, gamma 1 (formerly LAMB2)	LAMC1	3915	2.68	up
GO:0007155	cell adhesion	0,00413	lectin, galactoside-binding, soluble, 3 binding protein	LGALS3BP	3959	4.40	down
GO:0007155	cell adhesion	0,00413	metastasis suppressor 1	MTSS1	9788	2.51	down
GO:0007155	cell adhesion	0,00413	nidogen (enactin)	NID	4811	2.09	up
GO:0007155	cell adhesion	0,00413	plexin C1	PLXNC1	10154	4.34	down
GO:0007155	cell adhesion	0,00413	protein tyrosine phosphatase, non-receptor type substrate 1	PTPNS1	140885	2.66	down
GO:0007155	cell adhesion	0,00413	scavenger receptor class B, member 1	SCARB1	949	2.87	down
GO:0007155	cell adhesion	0,00413	sparc/osteonectin, cwcv and kazal-like domains proteoglycan (testican)	SPOCK	6695	3.05	down
GO:0007155	cell adhesion	0,00413	transforming growth factor, beta-induced, 68kDa	TGFBI	7045	5.33	up
GO:0007155	cell adhesion	0,00413	vinculin	VCL	7414	2.68	up
GO:0008151	cell growth and/or maintenance	0,00247	ADP-ribosyltransferase (NAD+; poly (ADP-ribose) polymerase)	ADPRT	142	2.32	down
GO:0008151	cell growth and/or maintenance	0,00247	v-fos FBJ murine osteosarcoma viral oncogene homolog	FOS	2353	2.77	down
GO:0008151	cell growth and/or maintenance	0,00247	immediate early response 3	IER3	8870	2.09	up
GO:0008151	cell growth and/or maintenance	0,00247	insulin-like growth factor binding protein 2, 36kDa	IGFBP2	3485	3.48	up
GO:0008151	cell growth and/or maintenance	0,00247	insulin-like growth factor binding protein 3	IGFBP3	3486	5.08	up
GO:0008151	cell growth and/or maintenance	0,00247	inhibin, beta A (activin A, activin AB alpha polypeptide)	INHBA	3624	3.71	up
GO:0008151	cell growth and/or maintenance	0,00247	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	KIT	3815	2.49	down
GO:0008151	cell growth and/or maintenance	0,00247	v-maf musculoaponeurotic fibrosarcoma oncogene homolog (avian)	MAF	4094	3.22	down
GO:0008151	cell growth and/or maintenance	0,00247	MCF.2 cell line derived transforming sequence	MCF2	4168	2.77	down
GO:0008151	cell growth and/or maintenance	0,00247	c-mer proto-oncogene tyrosine kinase	MERTK	10461	2.10	down
GO:0008151	cell growth and/or maintenance	0,00247	MAX interacting protein 1	MXI1	4601	2.57	down
GO:0008151	cell growth and/or maintenance	0,00247	protease, serine, 11 (IGF binding)	PRSS11	5654	3.69	up
GO:0008151	cell growth and/or maintenance	0,00247	pituitary tumor-transforming 1	PTTG1	9232	3.59	up
GO:0008151	cell growth and/or maintenance	0,00247	Ras-related GTP binding C	RRAGC	64121	2.05	down
GO:0008151	cell growth and/or maintenance	0,00247	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3C	SEMA3C	10512	3.28	down
GO:0008151	cell growth and/or maintenance	0,00247	stathmin 1/oncoprotein 18	STMN1	3925	2.09	down
GO:0007399	neurogenesis	0,02150	ceroid-lipofuscinosis, neuronal 2, late infantile (Jansky-Bielschowsky disease)	CLN2	1200	4.83	down
GO:0007399	neurogenesis	0,02150	endothelin receptor type B	EDNRB	1910	2.05	down
GO:0007399	neurogenesis	0,02150	fasciculation and elongation protein zeta 1 (zygin I)	FEZ1	9638	2.45	down
GO:0007399	neurogenesis	0,02150	glucose phosphate isomerase	GPI	2821	2.02	up
GO:0007399	neurogenesis	0,02150	inhibin, beta A (activin A, activin AB alpha polypeptide)	INHBA	3624	3.71	up
GO:0007399	neurogenesis	0,02150	metastasis suppressor 1	MTSS1	9788	2.51	down
GO:0007399	neurogenesis	0,02150	olfactomedin 1	OLFM1	10439	2.97	down
GO:0007399	neurogenesis	0,02150	sema domain, immunoglobulin domain (Ig), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 4C	SEMA4C	54910	2.22	down
GO:0007399	neurogenesis	0,02150	sema domain, transmembrane domain (TM), and cytoplasmic domain, (semaphorin) 6A	SEMA6A	57556	2.49	down
GO:0007399	neurogenesis	0,02150	serine (or cysteine) proteinase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium derived factor), member 1	SERPINF1	5176	2.87	up
GO:0007399	neurogenesis	0,02150	sparc/osteonectin, cwcv and kazal-like domains proteoglycan (testican)	SPOCK	6695	3.05	down
GO:0007399	neurogenesis	0,02150	very low density lipoprotein receptor	VLDLR	7436	3.16	down
GO:0007601	visual perception	0,03534	cadherin 3, type 1, P-cadherin (placental)	CDH3	1001	3.14	down
GO:0007601	visual perception	0,03534	crystallin, alpha B	CRYAB	1410	4.81	down
GO:0007601	visual perception	0,03534	fibrillin 1 (Marfan syndrome)	FBN1	2200	4.47	up
GO:0007601	visual perception	0,03534	c-mer proto-oncogene tyrosine kinase	MERTK	10461	2.10	down
GO:0007601	visual perception	0,03534	putative nuclear protein ORF1-FL49	ORF1-FL49	84418	2.96	down
GO:0007601	visual perception	0,03534	transforming growth factor, beta-induced, 68kDa	TGFBI	7045	5.33	up
GO:0007601	visual perception	0,03534	tissue inhibitor of metalloproteinase 3 (Sorsby fundus dystrophy, pseudoinflammatory)	TIMP3	7078	2.39	up
GO:0006096	glycolysis	0,00072	enolase 1, (alpha)	ENO1	2023	6.97	up
GO:0006096	glycolysis	0,00072	glucose phosphate isomerase	GPI	2821	2.02	up
GO:0006096	glycolysis	0,00072	hexokinase 2	HK2	3099	2.91	up
GO:0006096	glycolysis	0,00072	lactate dehydrogenase A	LDHA	3939	7.76	up
GO:0006096	glycolysis	0,00072	phosphoglycerate kinase 1	PGK1	5230	2.96	up
GO:0006096	glycolysis	0,00072	pyruvate kinase, muscle	PKM2	5315	3.11	up
GO:0043123	positive regulation of I-kappaB kinase/NF-kappaB cascade	0,02855	caspase 1, apoptosis-related cysteine protease (interleukin 1, beta, convertase)	CASP1	834	2.42	up
GO:0043123	positive regulation of I-kappaB kinase/NF-kappaB cascade	0,02855	FK506 binding protein 1A, 12kDa	FKBP1A	2280	2.51	up
GO:0043123	positive regulation of I-kappaB kinase/NF-kappaB cascade	0,02855	lectin, galactoside-binding, soluble, 1 (galectin 1)	LGALS1	3956	2.78	up

APPENDIX S6 (continued)

GO ID	Function Name	Corrected P-Value	Gene name	Gene symbol	LocusLink ID	DE	Up / down
GO:0043123	positive regulation of I-kappaB kinase/NF-kappaB cascade	0,02855	lipopolysaccharide-induced TNF factor	LITAF	9516	2.54	down
GO:0043123	positive regulation of I-kappaB kinase/NF-kappaB cascade	0,02855	receptor-interacting serine-threonine kinase 2	RIPK2	8767	2.20	up
GO:0043123	positive regulation of I-kappaB kinase/NF-kappaB cascade	0,02855	solute carrier family 20 (phosphate transporter), member 1	SLC20A1	6574	4.49	up
GO:0006396	RNA processing	0,00525	CUG triplet repeat, RNA binding protein 2	CUGBP2	10659	3.69	down
GO:0006396	RNA processing	0,00525	mitochondrial ribosomal protein L44	MRPL44	65080	3.90	down
GO:0006396	RNA processing	0,00525	poly(A) binding protein, cytoplasmic 4 (inducible form)	PABPC4	8761	2.47	down
GO:0006396	RNA processing	0,00525	putative ribonuclease III	RNASE3L	29102	2.21	up
GO:0006396	RNA processing	0,00525	Sjogren syndrome antigen B (autoantigen La)	SSB	6741	2.68	up
GO:0006396	RNA processing	0,00525	tRNA nucleotidyl transferase, CCA-adding, 1	TRNT1	51095	2.04	down
GO:0006916	anti-apoptosis	0,03115	apoptosis regulator bcl-x (bcl-2-like 1 protein)	BCL2L1	598	3.29	up
GO:0006916	anti-apoptosis	0,03115	immediate early response 3	IER3	8870	2.09	up
GO:0006916	anti-apoptosis	0,03115	secreted frizzled-related protein 1	SFRP1	6422	11.88	up
GO:0006916	anti-apoptosis	0,03115	synuclein, alpha (non A4 component of amyloid precursor)	SNCA	6622	2.53	down
GO:0006916	anti-apoptosis	0,03115	secreted phosphoprotein 1 (osteopontin, bone sialoprotein I, early T-lymphocyte activation 1)	SPP1	6696	9.34	down
GO:0007010	cytoskeleton organization and biogenesis	0,01532	adducin 1 (alpha)	ADD1	118	2.36	down
GO:0007010	cytoskeleton organization and biogenesis	0,01532	calponin 2	CNN2	1265	2.56	up
GO:0007010	cytoskeleton organization and biogenesis	0,01532	NEDD9 interacting protein with calponin homology and LIM domains	NICAL	64780	3.92	down
GO:0007010	cytoskeleton organization and biogenesis	0,01532	sema domain, transmembrane domain (TM), and cytoplasmic domain, (semaphorin) 6A	SEMA6A	57556	2.49	down
GO:0007010	cytoskeleton organization and biogenesis	0,01532	thymosin, beta 4, X-linked	TMSB4X	7114	15.85	up
GO:0006259	DNA metabolism	0,00081	DNase II-like acid DNase	DLAD	58511	5.09	down
GO:0006259	DNA metabolism	0,00081	karyopherin alpha 2 (RAG cohort 1, importin alpha 1)	KPNA2	3838	2.60	up
GO:0006259	DNA metabolism	0,00081	5'-nucleotidase, ecto (CD73)	NT5E	4907	15.09	up
GO:0006259	DNA metabolism	0,00081	pituitary tumor-transforming 1	PTTG1	9232	3.59	up
GO:0006897	endocytosis	0,04240	bridging integrator 3	BIN3	55909	2.30	down
GO:0006897	endocytosis	0,04240	growth hormone receptor	GHR	2690	2.13	down
GO:0006897	endocytosis	0,04240	low density lipoprotein receptor (familial hypercholesterolemia)	LDLR	3949	3.02	up
GO:0006897	endocytosis	0,04240	target of myb1 (chicken)	TOM1	10043	4.37	down
GO:0006897	endocytosis	0,04240	very low density lipoprotein receptor	VLDLR	7436	3.16	down
GO:0006869	lipid transport	0,01470	low density lipoprotein receptor (familial hypercholesterolemia)	LDLR	3949	3.02	up
GO:0006869	lipid transport	0,01470	oxysterol binding protein-like 9	OSBPL9	114883	2.69	down
GO:0006869	lipid transport	0,01470	phospholipid transfer protein	PLTP	5360	2.17	down
GO:0006869	lipid transport	0,01470	prosaposin (variant Gaucher disease and variant metachromatic leukodystrophy)	PSAP	5660	3.22	down
GO:0006869	lipid transport	0,01470	very low density lipoprotein receptor	VLDLR	7436	3.16	down
GO:0007605	perception of sound	0,04596	collagen, type I, alpha 2	COL1A2	1278	16.18	up
GO:0007605	perception of sound	0,04596	endothelin receptor type B	EDNRB	1910	2.05	down
GO:0007605	perception of sound	0,04596	microphthalmia-associated transcription factor	MITF	4286	4.22	down
GO:0007605	perception of sound	0,04596	peripheral myelin protein 22	PMP22	5376	4.15	down
GO:0007605	perception of sound	0,04596	SRY (sex determining region Y)-box 10	SOX10	6663	3.18	down
GO:0006464	protein modification	0,04622	beta-site APP-cleaving enzyme 2	BACE2	25825	3.61	down
GO:0006464	protein modification	0,04622	peptidylglycine alpha-amidating monooxygenase	PAM	5066	4.66	up
GO:0006464	protein modification	0,04622	procollagen-lysine, 2-oxoglutarate 5-dioxygenase (lysine hydroxylase, Ehlers-Danlos syndrome type VI)	PLOD	5351	2.68	up
GO:0006464	protein modification	0,04622	procollagen-lysine, 2-oxoglutarate 5-dioxygenase (lysine hydroxylase) 2	PLOD2	5352	7.69	up
GO:0019883	antigen presentation, endogenous antigen	0,00001	major histocompatibility complex, class I, A	HLA-A	3105	4.54	down
GO:0019883	antigen presentation, endogenous antigen	0,00001	major histocompatibility complex, class I, B	HLA-B	3106	3.21	down
GO:0019883	antigen presentation, endogenous antigen	0,00001	major histocompatibility complex, class I, C	HLA-C	3107	3.00	down
GO:0019883	antigen presentation, endogenous antigen	0,00001	major histocompatibility complex, class I, E	HLA-E	3133	2.96	down
GO:0019885	antigen processing, endogenous antigen via MHC class I	0,00018	major histocompatibility complex, class I, A	HLA-A	3105	4.54	down
GO:0019885	antigen processing, endogenous antigen via MHC class I	0,00018	major histocompatibility complex, class I, B	HLA-B	3106	3.21	down
GO:0019885	antigen processing, endogenous antigen via MHC class I	0,00018	major histocompatibility complex, class I, C	HLA-C	3107	3.00	down
GO:0019885	antigen processing, endogenous antigen via MHC class I	0,00018	major histocompatibility complex, class I, E	HLA-E	3133	2.96	down
GO:0030036	actin cytoskeleton organization and biogenesis	0,02056	filamin B, beta (actin binding protein 278)	FLNB	2317	2.66	up
GO:0030036	actin cytoskeleton organization and biogenesis	0,02056	metastasis suppressor 1	MTSS1	9788	2.51	down
GO:0030036	actin cytoskeleton organization and biogenesis	0,02056	syndecan binding protein (syntenin)	SDCBP	6386	2.67	down
GO:0030036	actin cytoskeleton organization and biogenesis	0,02056	spectrin, beta, non-erythrocytic 5	SPTBN5	51332	3.55	down
GO:0042981	regulation of apoptosis	0,04401	apoptosis regulator bcl-x (bcl-2-like 1 protein)	BCL2L1	598	3.29	up
GO:0042981	regulation of apoptosis	0,04401	caspase 1, apoptosis-related cysteine protease (interleukin 1, beta, convertase)	CASP1	834	2.42	up
GO:0042981	regulation of apoptosis	0,04401	receptor-interacting serine-threonine kinase 2	RIPK2	8767	2.20	up
GO:0042981	regulation of apoptosis	0,04401	reticulon 4	RTN4	57142	2.88	up
GO:0001558	regulation of cell growth	0,04376	insulin-like growth factor binding protein 2, 36kDa	IGFBP2	3485	3.48	up
GO:0001558	regulation of cell growth	0,04376	insulin-like growth factor binding protein 3	IGFBP3	3486	5.08	up
GO:0001558	regulation of cell growth	0,04376	protease, serine, 11 (IGF binding)	PRSS11	5654	3.69	up
GO:0001558	regulation of cell growth	0,04376	vascular endothelial growth factor B	VEGFB	7423	2.89	down
GO:0007169	transmembrane receptor protein tyrosine kinase signaling pathway	0,02855	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	KIT	3815	2.49	down
GO:0007169	transmembrane receptor protein tyrosine kinase signaling pathway	0,02855	myelin protein zero-like 1	MPZL1	9019	2.80	up

APPENDIX S6 (continued)

GO ID	Function Name	Corrected P-Value	Gene name	Gene symbol	LocusLink ID	DE	Up / down
GO:0007169	transmembrane receptor protein tyrosine kinase signaling pathway	0,02855	metastasis suppressor 1	MTSS1	9788	2.51	down
GO:0007411	axon guidance	0,00685	fasciculation and elongation protein zeta 1 (zygin I)	FEZ1	9638	2.45	down
GO:0007411	axon guidance	0,00685	sema domain, transmembrane domain (TM), and cytoplasmic domain, (semaphorin) 6A	SEMA6A	57556	2.49	down
GO:0007411	axon guidance	0,00685	spondin 2, extracellular matrix protein	SPON2	10417	2.73	up
GO:0006888	ER to Golgi transport	0,01330	BET1 homolog (S. cerevisiae)	BET1	10282	2.00	down
GO:0006888	ER to Golgi transport	0,01330	SEC22 vesicle trafficking protein-like 3 (S. cerevisiae)	SEC22L3	9117	2.23	down
GO:0006888	ER to Golgi transport	0,01330	SEC24 related gene family, member D (S. cerevisiae)	SEC24D	9871	2.10	up
GO:0006836	neurotransmitter transport	0,00872	amyotrophic lateral sclerosis 2 (juvenile) chromosome region, candidate 3	ALS2CR3	66008	2.27	down
GO:0006836	neurotransmitter transport	0,00872	solute carrier family 6 (neurotransmitter transporter, creatine), member 8	SLC6A8	6535	2.15	down
GO:0006605	protein targeting	0,02257	GABA(A) receptor-associated protein	GABARAP	11337	2.08	down
GO:0006605	protein targeting	0,02257	Hermansky-Pudlak syndrome 4	HPS4	89781	3.05	down
GO:0006605	protein targeting	0,02257	translocation associated membrane protein 2	TRAM2	9697	7.29	up
GO:0010038	response to metal ion	0,00019	metallothionein 1X	MT1X	4501	2.61	up
GO:0010038	response to metal ion	0,00019	neural precursor cell expressed, developmentally down-regulated 4-like	NEDD4L	23327	3.53	down
GO:0006805	xenobiotic metabolism	0,04728	cytochrome P450, family 3, subfamily A, polypeptide 4	CYP3A4	1576	3.63	down
GO:0006805	xenobiotic metabolism	0,04728	epoxide hydrolase 1, microsomal (xenobiotic)	EPHX1	2052	4.26	down
GO:0006805	xenobiotic metabolism	0,04728	NAD(P)H dehydrogenase, quinone 1	NQO1	1728	4.29	up
GO:0006835	dicarboxylic acid transport	0,00950	solute carrier family 1 (glutamate/neutral amino acid transporter), member 4	SLC1A4	6509	4.24	down
GO:0006835	dicarboxylic acid transport	0,00950	solute carrier family 1 (neutral amino acid transporter), member 5	SLC1A5	6510	4.79	up
GO:0000067	DNA replication and chromosome cycle	0,00275	centromere protein F, 350/400ka (mitosin)	CENPF	1063	2.44	up
GO:0000067	DNA replication and chromosome cycle	0,00275	pituitary tumor-transforming 1	PTTG1	9232	3.59	up
GO:0006268	DNA unwinding	0,00622	high mobility group AT-hook 1	HMGA1	3159	5.16	up
GO:0006726	eye pigment biosynthesis	0,00035	oculocutaneous albinism II (pink-eye dilution homolog, mouse)	OCA2	4948	4.29	down
GO:0000085	G2 phase of mitotic cell cycle	0,00036	karyopherin alpha 2 (RAG cohort 1, importin alpha 1)	KPNA2	3838	2.60	up
GO:0005978	glycogen biosynthesis	0,00940	glucan (1,4-alpha-), branching enzyme 1 (glycogen branching enzyme, Andersen disease, glycogen storage disease type IV)	GBE1	2632	4.56	up
GO:0005978	glycogen biosynthesis	0,00940	glycogenin 2	GYG2	8908	3.09	down
GO:0006687	glycosphingolipid metabolism	0,00125	GM2 ganglioside activator protein	GM2A	2760	3.46	down
GO:0006687	glycosphingolipid metabolism	0,00125	prosaposin (variant Gaucher disease and variant metachromatic leukodystrophy)	PSAP	5660	3.22	down
GO:0006183	GTP biosynthesis	0,01304	guanylate kinase 1	GUK1	2987	2.76	down
GO:0006183	GTP biosynthesis	0,01304	non-metastatic cells 1, protein (NM23A) expressed in	NME1	4830	3.12	up
GO:0007599	hemostasis	0,00503	glucose phosphate isomerase	GPI	2821	2.02	up
GO:0007599	hemostasis	0,00503	Hermansky-Pudlak syndrome 4	HPS4	89781	3.05	down
GO:0007611	learning and/or memory	0,00629	S100 calcium binding protein, beta (neural)	S100B	6285	2.34	down
GO:0006345	loss of chromatin silencing	0,00035	high mobility group AT-hook 1	HMGA1	3159	5.16	up
GO:0007040	lysosome organization and biogenesis	0,00270	Hermansky-Pudlak syndrome 4	HPS4	89781	3.05	down
GO:0007040	lysosome organization and biogenesis	0,00270	prosaposin (variant Gaucher disease and variant metachromatic leukodystrophy)	PSAP	5660	3.22	down
GO:0000072	M phase specific microtubule process	0,00000	karyopherin alpha 2 (RAG cohort 1, importin alpha 1)	KPNA2	3838	2.60	up
GO:0006583	melanin biosynthesis from tyrosine	0,00511	dopachrome tautomerase (dopachrome delta-isomerase, tyrosine-related protein 2)	DCT	1638	22.42	down
GO:0006583	melanin biosynthesis from tyrosine	0,00511	tyrosinase-related protein 1	TYRP1	7306	3.95	down
GO:0006509	membrane protein ectodomain proteolysis	0,00121	a disintegrin and metalloproteinase domain 10	ADAM10	102	2.46	down
GO:0006509	membrane protein ectodomain proteolysis	0,00121	beta-site APP-cleaving enzyme 2	BACE2	25825	3.61	down
GO:0046785	microtubule polymerization	0,01498	tubulin, alpha 1 (testis specific)	TUBA1	7277	2.01	up
GO:0007194	negative regulation of adenylate cyclase activity	0,01331	endothelin receptor type B	EDNRB	1910	2.05	down
GO:0007194	negative regulation of adenylate cyclase activity	0,01331	guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide 3	GNAI3	2773	2.21	up
GO:0007162	negative regulation of cell adhesion	0,01511	a disintegrin and metalloproteinase domain 10	ADAM10	102	2.46	down
GO:0007162	negative regulation of cell adhesion	0,01511	transforming growth factor, beta-induced, 68kDa	TGFBI	7045	5.33	up
GO:0015804	neutral amino acid transport	0,00123	solute carrier family 1 (glutamate/neutral amino acid transporter), member 4	SLC1A4	6509	4.24	down
GO:0015804	neutral amino acid transport	0,00123	solute carrier family 1 (neutral amino acid transporter), member 5	SLC1A5	6510	4.79	up
GO:0006607	NLS-bearing substrate-nucleus import	0,02205	karyopherin alpha 2 (RAG cohort 1, importin alpha 1)	KPNA2	3838	2.60	up
GO:0006337	nucleosome disassembly	0,00117	high mobility group AT-hook 1	HMGA1	3159	5.16	up
GO:0006493	O-linked glycosylation	0,01317	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 1 (GalNAc-T1)	GALNT1	2589	3.21	up
GO:0006493	O-linked glycosylation	0,01317	low density lipoprotein receptor (familial hypercholesterolemia)	LDLR	3949	3.02	up
GO:0001503	ossification	0,03726	secreted protein, acidic, cysteine-rich (osteonectin)	SPARC	6678	2.52	up
GO:0001503	ossification	0,03726	secreted phosphoprotein 1 (osteopontin, bone sialoprotein I, early T-lymphocyte activation 1)	SPP1	6696	9.34	down
GO:0045807	positive regulation of endocytosis	0,00000	neural precursor cell expressed, developmentally down-regulated 4-like	NEDD4L	23327	3.53	down
GO:0045941	positive regulation of transcription	0,04815	high mobility group AT-hook 1	HMGA1	3159	5.16	up
GO:0000018	regulation of DNA recombination	0,00119	karyopherin alpha 2 (RAG cohort 1, importin alpha 1)	KPNA2	3838	2.60	up
GO:0008016	regulation of heart rate	0,04842	CUG triplet repeat, RNA binding protein 2	CUGBP2	10659	3.69	down
GO:0008016	regulation of heart rate	0,04842	tropomyosin 1 (alpha)	TPM1	7168	2.22	up
GO:0007088	regulation of mitosis	0,01959	cell division cycle 2-like 5 (cholinesterase-related cell division controller)	CDC2L5	8621	3.28	down
GO:0007088	regulation of mitosis	0,01959	centromere protein F, 350/400ka (mitosin)	CENPF	1063	2.44	up
GO:0006937	regulation of muscle contraction	0,01975	myosin, light polypeptide 9, regulatory	MYL9	10398	3.40	up
GO:0006937	regulation of muscle contraction	0,01975	tropomyosin 1 (alpha)	TPM1	7168	2.22	up
GO:0042176	regulation of protein catabolism	0,00127	neural precursor cell expressed, developmentally down-regulated 4-like	NEDD4L	23327	3.53	down

APPENDIX S6 (continued)

GO ID	Function Name	Corrected P-Value	Gene name	Gene symbol	LocusLink ID	DE	Up / down
GO:0009636	response to toxin	0,00930	epoxide hydrolase 1, microsomal (xenobiotic)	EPHX1	2052	4.26	down
GO:0009636	response to toxin	0,00930	NAD(P)H dehydrogenase, quinone 1	NQO1	1728	4.29	up
GO:0006883	sodium ion homeostasis	0,00038	neural precursor cell expressed, developmentally down-regulated 4-like	NEDD4L	23327	3.53	down
GO:0016126	sterol biosynthesis	0,00037	sterol-C4-methyl oxidase-like	SC4MOL	6307	2.50	up
GO:0016126	sterol biosynthesis	0,00037	squalene epoxidase	SQLE	6713	2.10	up
GO:0006383	transcription from Pol III promoter	0,02221	polymerase (RNA) II (DNA directed) polypeptide L, 7.6kDa	POLR2L	5441	3.89	up
GO:0006383	transcription from Pol III promoter	0,02221	Sjogren syndrome antigen B (autoantigen La)	SSB	6741	2.68	up
GO:0030104	water homeostasis	0,00037	neural precursor cell expressed, developmentally down-regulated 4-like	NEDD4L	23327	3.53	down
GO:0006930	"substrate-bound cell migration, cell extension"	0,02385	syndecan binding protein (syntenin)	SDCBP	6386	2.67	down
GO:0007271	"synaptic transmission, cholinergic"	0,03118	NAD(P)H dehydrogenase, quinone 1	NQO1	1728	4.29	up
GO:0009308	amine metabolism	0,01349	sulfotransferase family, cytosolic, 1C, member 1	SULT1C1	6819	2.26	up
GO:0008637	apoptotic mitochondrial changes	0,03330	apoptosis regulator bcl-x (bcl-2-like 1 protein)	BCL2L1	598	3.29	up
GO:0006527	arginine catabolism	0,03158	dimethylarginine dimethylaminohydrolase 1	DDAH1	23576	2.30	up
GO:0006421	asparaginyl-tRNA aminoacylation	0,00690	hypothetical protein FLJ23441	FLJ23441	79731	2.42	down
GO:0006422	aspartyl-tRNA aminoacylation	0,03220	hypothetical protein FLJ23441	FLJ23441	79731	2.42	down
GO:0048143	astrocyte activation	0,00000	S100 calcium binding protein, beta (neural)	S100B	6285	2.34	down
GO:0006200	ATP catabolism	0,00000	ATP citrate lyase	ACLY	47	2.00	up
GO:0000917	barrier septum formation	0,00000	bridging integrator 3	BIN3	55909	2.30	down
GO:0042113	B-cell activation	0,03138	immunoglobulin (CD79A) binding protein 1	IGBP1	3476	2.78	down
GO:0016477	cell migration	0,03242	fibronectin 1	FN1	2335	69.37	up
GO:0007623	circadian rhythm	0,04349	heme binding protein 1	HEBP1	50865	2.25	down
GO:0006101	citrate metabolism	0,00624	ATP citrate lyase	ACLY	47	2.00	up
GO:0006763	coenzyme A metabolism	0,00000	ATP citrate lyase	ACLY	47	2.00	up
GO:0006878	copper ion homeostasis	0,02243	metallothionein 2A	MT2A	4502	2.08	up
GO:0009214	cyclic nucleotide catabolism	0,00000	2',3'-cyclic nucleotide 3' phosphodiesterase	CNP	1267	3.01	down
GO:0042994	cytoplasmic sequestering of transcription factor	0,00749	MAX interacting protein 1	MXI1	4601	2.57	down
GO:0009157	deoxyribonucleoside monophosphate biosynthesis	0,00000	thymidylate synthetase	TYMS	7298	3.13	up
GO:0006304	DNA modification	0,01411	nucleoside phosphorylase	NP	4860	2.03	up
GO:0006231	dTMP biosynthesis	0,00000	thymidylate synthetase	TYMS	7298	3.13	up
GO:0001701	embryonic development (sensu Mammalia)	0,03353	a disintegrin and metalloproteinase domain 10	ADAM10	102	2.46	down
GO:0007492	endoderm development	0,00709	laminin, gamma 1 (formerly LAMB2)	LAMC1	3915	2.68	up
GO:0006112	energy reserve metabolism	0,03179	S100 calcium binding protein, beta (neural)	S100B	6285	2.34	down
GO:0030218	erythrocyte differentiation	0,01478	inhibin, beta A (activin A, activin AB alpha polypeptide)	INHBA	3624	3.71	up
GO:0006636	fatty acid desaturation	0,01398	fatty acid desaturase 3	FADS3	3995	2.11	up
GO:0019395	fatty acid oxidation	0,02260	adiponectin receptor 1	ADIPOR1	51094	2.15	down
GO:0001574	ganglioside biosynthesis	0,00000	sialyltransferase 9 (CMP-NeuAc:lactosylceramide alpha-2,3-sialyltransferase; GM3 synthase)	SIAT9	8869	2.14	down
GO:0007281	germ cell development	0,02162	CUG triplet repeat, RNA binding protein 1	CUGBP1	10658	2.65	down
GO:0006543	glutamine catabolism	0,00000	glutaminase	GLS	2744	2.07	up
GO:0019377	glycolipid catabolism	0,00000	GM2 ganglioside activator protein	GM2A	2760	3.46	down
GO:0006027	glycosaminoglycan catabolism	0,03263	glucuronidase, beta	GUSB	2990	3.56	down
GO:0007150	growth pattern	0,00610	growth hormone receptor	GHR	2690	2.13	down
GO:0042541	hemoglobin biosynthesis	0,00699	inhibin, beta A (activin A, activin AB alpha polypeptide)	INHBA	3624	3.71	up
GO:0008334	histone mRNA metabolism	0,00000	Sjogren syndrome antigen B (autoantigen La)	SSB	6741	2.68	up
GO:0009755	hormone mediated signaling	0,03199	adiponectin receptor 1	ADIPOR1	51094	2.15	down
GO:0048151	hyperphosphorylation	0,00000	S100 calcium binding protein, beta (neural)	S100B	6285	2.34	down
GO:0030595	immune cell chemotaxis	0,01464	secreted phosphoprotein 1 (osteopontin, bone sialoprotein I, early T-lymphocyte activation 1)	SPP1	6696	9.34	down
GO:0050930	induction of positive chemotaxis	0,01522	secreted phosphoprotein 1 (osteopontin, bone sialoprotein I, early T-lymphocyte activation 1)	SPP1	6696	9.34	down
GO:0008610	lipid biosynthesis	0,02404	ATP citrate lyase	ACLY	47	2.00	up
GO:0007515	lymph gland development	0,00000	vascular endothelial growth factor C	VEGFC	7424	2.12	up
GO:0007638	mechanosensory behavior	0,02294	peripheral myelin protein 22	PMP22	5376	4.15	down
GO:0030318	melanocyte differentiation	0,00000	microphthalmia-associated transcription factor	MITF	4286	4.22	down
GO:0007613	memory	0,00000	very low density lipoprotein receptor	VLDLR	7436	3.16	down
GO:0030035	microspike biogenesis	0,00000	metastasis suppressor 1	MTSS1	9788	2.51	down
GO:0030033	microvillus biogenesis	0,00000	FXYD domain containing ion transport regulator 5	FXYD5	53827	2.01	up
GO:0007094	mitotic spindle checkpoint	0,02178	MAD2 mitotic arrest deficient-like 2 (yeast)	MAD2L2	10459	2.28	down
GO:0000022	mitotic spindle elongation	0,00728	protein regulator of cytokinesis 1	PRC1	9055	3.16	up
GO:0030224	monocyte differentiation	0,00000	interferon, gamma-inducible protein 16	IFI16	3428	2.15	down
GO:0006376	mRNA splice site selection	0,02348	CUG triplet repeat, RNA binding protein 1	CUGBP1	10658	2.65	down
GO:0007518	myoblast cell fate determination	0,00664	myelin expression factor 2	MYEF2	50804	2.73	down
GO:0042985	negative regulation of amyloid precursor protein biosynthesis	0,00000	beta-site APP-cleaving enzyme 2	BACE2	25825	3.61	down
GO:0016525	negative regulation of angiogenesis	0,04297	serine (or cysteine) proteinase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium derived factor), member 1	SERPINF1	5176	2.87	up
GO:0019987	negative regulation of anti-apoptosis	0,00000	reticulon 4	RTN4	57142	2.88	up
GO:0030517	negative regulation of axon extension	0,00000	reticulon 4	RTN4	57142	2.88	up
GO:0045578	negative regulation of B-cell differentiation	0,00603	inhibin, beta A (activin A, activin AB alpha polypeptide)	INHBA	3624	3.71	up

APPENDIX S6 (continued)

GO ID	Function Name	Corrected P-Value	Gene name	Gene symbol	LocusLink ID	DE	Up / down
GO:0030502	negative regulation of bone mineralization	0,01373	secreted phosphoprotein 1 (osteopontin, bone sialoprotein I, early T-lymphocyte activation 1)	SPP1	6696	9.34	down
GO:0046588	negative regulation of calcium-dependent cell-cell adhesion	0,00000	FXYD domain containing ion transport regulator 5	FXYD5	53827	2.01	up
GO:0046882	negative regulation of follicle-stimulating hormone secretion	0,02226	inhibin, beta A (activin A, activin AB alpha polypeptide)	INHBA	3624	3.71	up
GO:0045077	negative regulation of interferon-gamma biosynthesis	0,00648	inhibin, beta A (activin A, activin AB alpha polypeptide)	INHBA	3624	3.71	up
GO:0045650	negative regulation of macrophage differentiation	0,00596	inhibin, beta A (activin A, activin AB alpha polypeptide)	INHBA	3624	3.71	up
GO:0042326	negative regulation of phosphorylation	0,00718	inhibin, beta A (activin A, activin AB alpha polypeptide)	INHBA	3624	3.71	up
GO:0017148	negative regulation of protein biosynthesis	0,04376	eukaryotic translation initiation factor 4E binding protein 3	EIF4EBP3	8637	2.11	down
GO:0009968	negative regulation of signal transduction	0,00681	insulin-like growth factor binding protein 3	IGFBP3	3486	5.08	up
GO:0008634	negative regulation of survival gene product activity	0,02312	apoptosis regulator bcl-x (bcl-2-like 1 protein)	BCL2L1	598	3.29	up
GO:0045947	negative regulation of translational initiation	0,00640	eukaryotic translation initiation factor 4E binding protein 3	EIF4EBP3	8637	2.11	down
GO:0007528	neuromuscular junction development	0,01314	CUG triplet repeat, RNA binding protein 2	CUGBP2	10659	3.69	down
GO:0001504	neurotransmitter uptake	0,01303	solute carrier family 6 (neurotransmitter transporter, creatine), member 8	SLC6A8	6535	2.15	down
GO:0007263	nitric oxide mediated signal transduction	0,02366	dimethylarginine dimethylaminohydrolase 1	DDAH1	23576	2.30	up
GO:0007220	Notch receptor processing	0,00000	a disintegrin and metalloproteinase domain 10	ADAM10	102	2.46	down
GO:0006913	nucleocytoplasmic transport	0,00632	a disintegrin and metalloproteinase domain 10	ADAM10	102	2.46	down
GO:0009142	nucleoside triphosphate biosynthesis	0,01507	non-metastatic cells 1, protein (NM23A) expressed in	NME1	4830	3.12	up
GO:0009166	nucleotide catabolism	0,00617	5'-nucleotidase, ecto (CD73)	NT5E	4907	15.09	up
GO:0009312	oligosaccharide biosynthesis	0,02194	UDP-Gal:betaGlcNAc beta 1,4- galactosyltransferase, polypeptide 1	B4GALT1	2683	4.63	up
GO:0001541	ovarian follicle development	0,01493	inhibin, beta A (activin A, activin AB alpha polypeptide)	INHBA	3624	3.71	up
GO:0030913	paranodal junction formation	0,00000	CD9 antigen (p24)	CD9	928	2.26	down
GO:0006098	pentose-phosphate shunt	0,02210	3-hydroxyisobutyrate dehydrogenase	HIBADH	11112	2.23	down
GO:0001519	peptide amidation	0,00000	peptidylglycine alpha-amidating monooxygenase	PAM	5066	4.66	up
GO:0016486	peptide hormone processing	0,01337	beta-site APP-cleaving enzyme 2	BACE2	25825	3.61	down
GO:0006518	peptide metabolism	0,01385	peptidylglycine alpha-amidating monooxygenase	PAM	5066	4.66	up
GO:0045917	positive regulation of complement activation	0,00000	S100 calcium binding protein, beta (neural)	S100B	6285	2.34	down
GO:0048081	positive regulation of cuticle pigmentation	0,00000	Hermansky-Pudlak syndrome 4	HPS4	89781	3.05	down
GO:0043085	positive regulation of enzyme activity	0,00672	anterior pharynx defective 1B-like	PSFL	83464	2.18	down
GO:0048075	positive regulation of eye pigmentation	0,00000	Hermansky-Pudlak syndrome 4	HPS4	89781	3.05	down
GO:0046881	positive regulation of follicle-stimulating hormone secretion	0,01437	inhibin, beta A (activin A, activin AB alpha polypeptide)	INHBA	3624	3.71	up
GO:0045663	positive regulation of myoblast differentiation	0,00000	insulin-like growth factor binding protein 3	IGFBP3	3486	5.08	up
GO:0050769	positive regulation of neurogenesis	0,00000	serine (or cysteine) proteinase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium derived factor), member 1	SERPINF1	5176	2.87	up
GO:0042102	positive regulation of T-cell proliferation	0,02277	secreted phosphoprotein 1 (osteopontin, bone sialoprotein I, early T-lymphocyte activation 1)	SPP1	6696	9.34	down
GO:0006693	prostaglandin metabolism	0,03285	hydroxyprostaglandin dehydrogenase 15-(NAD)	HPGD	3248	2.68	down
GO:0016579	protein deubiquitination	0,00739	ubiquitin carboxyl-terminal esterase L1 (ubiquitin thiolesterase)	UCHL1	7345	2.09	up
GO:0008104	protein localization	0,04403	bridging integrator 3	BIN3	55909	2.30	down
GO:0016485	protein processing	0,04272	anterior pharynx defective 1B-like	PSFL	83464	2.18	down
GO:0050821	protein stabilization	0,04221	Hermansky-Pudlak syndrome 4	HPS4	89781	3.05	down
GO:0006612	protein-membrane targeting	0,02330	syndecan binding protein (syntenin)	SDCBP	6386	2.67	down
GO:0042035	regulation of cytokine biosynthesis	0,00000	S100 calcium binding protein, beta (neural)	S100B	6285	2.34	down
GO:0048169	regulation of long-term neuronal synaptic plasticity	0,00000	S100 calcium binding protein, beta (neural)	S100B	6285	2.34	down
GO:0045637	regulation of myeloid blood cell differentiation	0,00000	secreted phosphoprotein 1 (osteopontin, bone sialoprotein I, early T-lymphocyte activation 1)	SPP1	6696	9.34	down
GO:0006356	regulation of transcription from Pol I promoter	0,01424	polymerase (RNA) II (DNA directed) polypeptide L, 7.6kDa	POLR2L	5441	3.89	up
GO:0009628	response to abiotic stimulus	0,00000	stannin	SNN	8303	2.29	down
GO:0009607	response to biotic stimulus	0,04246	immunoglobulin (CD79A) binding protein 1	IGBP1	3476	2.78	down
GO:0009409	response to cold	0,01361	guanosine monophosphate reductase	GMPR	2766	3.10	down
GO:0009605	response to external stimulus	0,04323	inhibin, beta A (activin A, activin AB alpha polypeptide)	INHBA	3624	3.71	up
GO:0016246	RNA interference	0,00589	CUG triplet repeat, RNA binding protein 1	CUGBP1	10658	2.65	down
GO:0006405	RNA-nucleus export	0,01451	Sjogren syndrome antigen B (autoantigen La)	SSB	6741	2.68	up
GO:0030149	sphingolipid catabolism	0,00760	GM2 ganglioside activator protein	GM2A	2760	3.46	down
GO:0006929	substrate-bound cell migration	0,00000	vascular endothelial growth factor C	VEGFC	7424	2.12	up
GO:0042088	T-helper 1 type immune response	0,00656	secreted phosphoprotein 1 (osteopontin, bone sialoprotein I, early T-lymphocyte activation 1)	SPP1	6696	9.34	down
GO:0006369	transcription termination from Pol II promoter	0,00000	MYC-associated zinc finger protein (purine-binding transcription factor)	MAZ	4150	2.54	down
GO:0007089	traversing start control point of mitotic cell cycle	0,03098	cell division cycle 2, G1 to S and G2 to M	CDC2	983	2.61	up
GO:0042780	tRNA 3'-processing	0,00000	tRNA nucleotidyl transferase, CCA-adding, 1	TRNT1	51095	2.04	down
GO:0006400	tRNA modification	0,01325	Sjogren syndrome antigen B (autoantigen La)	SSB	6741	2.68	up
GO:0009826	unidimensional cell growth	0,00000	bridging integrator 3	BIN3	55909	2.30	down
GO:0007034	vacuolar transport	0,00000	vacuolar protein sorting 41 (yeast)	VPS41	27072	3.05	down
GO:0006573	valine metabolism	0,00000	3-hydroxyisobutyrate dehydrogenase	HIBADH	11112	2.23	down

Knockdown of the endogenous NF1 gene (product): on the role in epidermal melanocyte proliferation and cell cycle regulation

Article IV

RNAi-mediated suppression of endogenous neurofibromin does not modulate ERK1/2 phosphorylation or the cell cycle in cultured primary human epidermal melanocytes.

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(article in preparation)

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Summary

Neurofibromin, gene product of the NF1 tumor suppressor gene, has been assigned a role in the regulation of cell proliferation as its central GTPase-activating protein (GAP)-related domain (GRD) is able to inhibit $p21^{Ras}$ activation. This results in subsequent inhibition of cell proliferation, manifested through cell cycle arrest. Via siRNA-mediated RNA interference (RNAi), we have investigated the effects of suppression of endogenous neurofibromin on cell cycle distribution and proliferative capacities of primary human epidermal melanocytes. We did not observe any changes in the proportion of melanocytes reaching the S and / or G2/M phase of the cell cycle, nor did we find any changes in cell proliferation in NF1 gene-silenced melanocytes. Furthermore, ERK phosphorylation (downstream $p21^{Ras}$ effector) did not increase upon neurofibromin suppression, nor did cyclin D1 expression. Our data demonstrates that neurofibromin does not seem to play a major role in cell cycle regulation in human epidermal melanocytes, at least in vitro and that $p21^{Ras}$ signaling, at least in part, is intact in these cells.

Key words: cell cycle, melanocyte, neurofibromin, proliferation, siRNA

Introduction

The human NF1 gene is located on chromosome 17q11.2 (Viskochil et al., 1993) and encodes a large 2818 aa protein, called neurofibromin (DeClue et al., 1991; Gutmann et al., 1991). Sequence analysis of neurofibromin revealed that a central region of around 360 aa shows significant sequence homology to the known catalytic domains of the mammalian $p21^{Ras}$ GTPase-activating protein $p120^{GAP}$, which interacts with $p21^{Ras}$, promotes hydrolysis of $p21^{Ras}$-bound GTP (active form) to GDP (inactive form) and results in inactivation of the $p21^{Ras}$ protein (Xu et al., 1990). Accordingly, loss and / or mutations of neurofibromin elevate $p21^{Ras}$ activity and are followed by activation of various $p21^{Ras}$ effectors. (Hyper)activation of $p21^{Ras}$ has been considered to be the causative event for tumor formation and other clinical manifestations in e.g. Neurofibromatosis type 1 (NF1) patients (Cichowski and Jacks, 2001). In three neurofibrosarcoma cell lines with decreased neurofibromin levels an increase in GTP-bound, activated $p21^{Ras}$ was observed (Basu et al., 1992; DeClue et al., 1992). In cultured melanocytes from NF1 patients (with a 50% reduction of neurofibromin levels due to haplo-insufficiency of the NF1 gene locus) no altered $p21^{Ras}$-GTP levels could be observed (Griesser et al., 1995). Control of the cell division cycle is at the heart of many biological processes in which inactivation of critical cell cycle regulators leads to uncontrolled cell proliferation and tumorigenesis. In primary rat Schwann cell cultures from $Nf1^{-/-}$ dorsal root ganglia an increased expression of the proto-oncogene cyclin D1 was observed, which is a positive regulator of the cell cycle promoting transition from the resting G1 phase into the DNA synthesizing S phase (Kim et al., 2001).

In order to further elucidate the role of neurofibromin, we decided to reduce the expression of the NF1 gene and

its corresponding gene product neurofibromin by applying RNA interference in cultured primary human epidermal melanocytes. In a next step, the effects of NF1 gene silencing on cell cycle distribution and cell proliferation were analyzed in this study.

Results

As neurofibromin is known as a tumor suppressor protein involved in regulation of cell proliferation through negative regulation of $p21^{Ras}$ activity, we wanted to examine the role of the *NF1* tumor suppressor gene (product) in cell growth. We attempted to reduce the expression of neurofibromin in primary human epidermal melanocytes by an RNAi-based strategy. After introduction of a siRNA duplex targeting the boundary of exon 44 and exon 45 of the *NF1* gene, the endogenous *NF1* mRNA levels as detected by qPCR were significantly reduced to 13% of baseline levels by day 2, but then slightly increased again to ~50% of baseline levels at day 8 (see figure 1a). The corresponding neurofibromin levels as detected by western blot analysis (using NF1(D) sc-67 and NF1(N) sc-68 antibodies) dropped equally fast down to 50%-40% of the baseline levels after 2 days and even further down to only 15% at day 4, remaining so until day 8 (see figure 1b). The levels of phosphorylated ERK1/2 (indicative for an increased MAPK pathway) and cyclin D1 did not change upon neurofibromin suppression. Using the MTS cell proliferation assay, no differences in cell proliferation were observed when neurofibromin was suppressed in epidermal melanocytes compared to siSCRAMBLE electroporated control melanocytes (see figure 1c), even after omission of growth factors (data not shown). As tight regulation of cell division, culminating in two daughter cells, is an important determinant of cell proliferation, we analysed the cell cycle distribution of epidermal melanocytes after suppression of neurofibromin levels (see figure 1d). Using flow cytometric analysis, the results show no significant increased proportion of melanocytes in the S and / or G_2 phases of the cell cycle upon neurofibromin depletion. However, a clear drop increase in G1 and concomitant decrease in S and G2 phase were observed probably due to increasing growth arrest caused by a rise in cell culture confluency over the 8 day time period. The results suggest that functional neurofibromin does not regulate the transitions from G_1 to S as well as from G_2 to M phase of the cell cycle, at least in epidermal melanocytes.

Discussion

In this study we present the effects of reduced neurofibromin on the proliferative capacities and cell cycle distribution of primary human epidermal melanocytes. We have used the RNA interference technology to knock-down the endogenous expression of NF1 mRNA and its corresponding protein product. Other tumor suppressor proteins like p53 and retinoblastoma are known to be involved in negative regulation of cell cycle, halting cells to enter the S phase (Galderisi et al., 2003). Moreover, it has been shown that cooperativity between mutations in Nf1 and p53 prolongs sympathetic neuron survival and proliferation (Vogel and Parada, 1998) or gives cell growth advantage to mice astrocytes (Bajenaru et al., 2001). Several lines of evidence have indicated that $p120^{GAP}$ and neurofibromin regulate $p21^{Ras}$ differently despite their common $p21^{Ras}$-GAP activity (Bollag and McCormick, 1991). It has already been suggested that neurofibromin might not be the major regulator of the $p21^{Ras}$ pathway. In cultured melanocytes from NF1 patients no altered $p21^{Ras}$-GTP levels could be observed indicating that melanocytes are capable of regulating their levels independently of the NF1 gene defects (Griesser et al., 1995). If neurofibromin suppresses cell growth by arresting cells in G0 or G1, its expression might be regulated in a cell cycle-dependent fashion. Neurofibromin expression in RAT-1A rat fibroblasts was shown to be upregulated after contact inhibition-induced G0/G1 growth arrest, while it did not show any changes when cells progressed through the cell cycle. This indicates that neurofibromin expression is a late event associated with cell growth arrest which may contribute to maintenance of the differentiated state (Norton et al., 1996). However, in cultured epidermal melanocytes from NF1 patients, decreased neurofibromin levels were shown to increase cell doubling times (indicative for decreased cell proliferation) and to induce differentiation in comparison to those from normal

patients (Kaufmann et al., 1991). An explanation for this paradoxical growth disadvantage in cultured NF1 melanocytes is currently unknown. Perhaps melanocytes belong to the group of neural crest cells where differentiation rather than proliferation is induced by $p21^{Ras}$ activation (Guerrero et al., 1986). However, some groups did not demonstrate any induction of proliferation or differentiation in cultured NF1 melanocytes (Abdel-Malek et al., 1993). This is in accordance to the results demonstrated in our study where we have silenced endogenous neurofibromin levels of normal epidermal melanocytes and did not see any changes in cell proliferation. In vivo however, an increase in the epidermal melanocyte population is found in NF1 patients compared to normal controls (Frenk and Marazzi, 1984; own observations), which suggests that probably impaired micro-environmental cues, cellular interactions and / or tissue organization are responsible for increased melanocyte proliferation and differentiation in NF1 skin.

In our study we have suppressed the endogenous expression levels of neurofibromin in normal human epidermal melanocytes and demonstrated that transient reduction of the NF1 gene product does not give any growth advantage to melanocytes monocultures nor does it significantly change the proportion of cells reaching the S and G2/M phase of the cell cycle. These results suggest that the function of neurofibromin is not limited to the regulation of the cell cycle, at least in human epidermal melanocytes. Melanocyte cultures prove to be a valuable system for molecular biological studies on the function of the NF1 gene (product). The use of in vivo mimicking co-culture systems in a two-dimensional or three-dimensional setting could further help in elucidating the interplay between (defective) neurofibromin and the micro-environment. This would teach us more on the relevant role of neurofibromin in proliferation and differentiation of normal human epidermal melanocytes in the skin.

Material & Methods

Human primary epidermal melanocyte cultures were established as described previously (Naeyaert et al., 1991; Boucneau et al., 2005). Human primary dermal fibroblasts were cultured in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FCS. A 25-base *NF1*-specific STEALTH^{TM} siRNA duplex (Invitrogen, Carlsbad, CA, USA) (5'-GCGCUGCUUCUUACUGUUC-UAGCUA-3' and its complement) and a siRNA scrambled control were used (5'-GCACAGUAAGA-GUGACGUCACGAUU-3' and its complement). 100-500 nM duplex was electroporated into primary epidermal melanocytes and dermal fibroblasts using the Neonatal Normal Human Epidermal Melanocytes kit (NHEM-Neo) and Neonatal Normal Human Dermal Fibroblast kit (NHEM, respectively (Amaxa, Germany). Quantitative Real-Time PCR analysis was performed as previously described using the appropriate primers (Boucneau et al., 2005). Western blot analysis using 20 µg of protein was performed according to standard procedures using polyclonal anti-neurofibromin antibody NF1(D) sc-67, NF1(N) sc-68, cyclin D1 (M-20) sc-718 (Santa Cruz Biotechnology, CA, USA), phospho-p42/44 MAP kinase (Thr202 / Tyr204) (Cell Signaling Technology), and anti-α-tubulin antibody (Sigma, St Louis, MO, USA). Immunocomplexes were visualized by chemiluminescence using the ECL+Plus system (Amersham, Uppsala, Sweden). For cell cycle analysis cells were harvested and resuspended in 1 ml PBS (Ca^{++} / Mg^{++} free, pH 7.4) and fixed overnight with 3 ml cold 70% ethanol at 4°C. After washing in PBS, 50 *µ*g/ml propidium iodide in PBS and 10 *µ*g/ml RNase A were added and incubated for 30 min at 37°C. Cell cycle analysis was performed by flow cytometry (FACS Calibur; Becton Dickinson). The proportion of cells in the G_1, S, G_2/M phases of the cell cycle was estimated using Cylchred software (University of Wales, College of Medicine). MTS cell proliferation assay was performed using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI, USA). Twenty microliters of CellTiter 96® Aqueous One Solution reagent (MTS tetrazolium compound) was added to each well of a 96-well culture plate containing siNF1 (100 nM) electroporated primary human epidermal melanocytes in 100 µL of culture medium. The plate was incubated for 2h at 37°C in 10% CO_2. The absorbance at 492 nm was measured using a Labsystems MultiSkan RC microplate reader.

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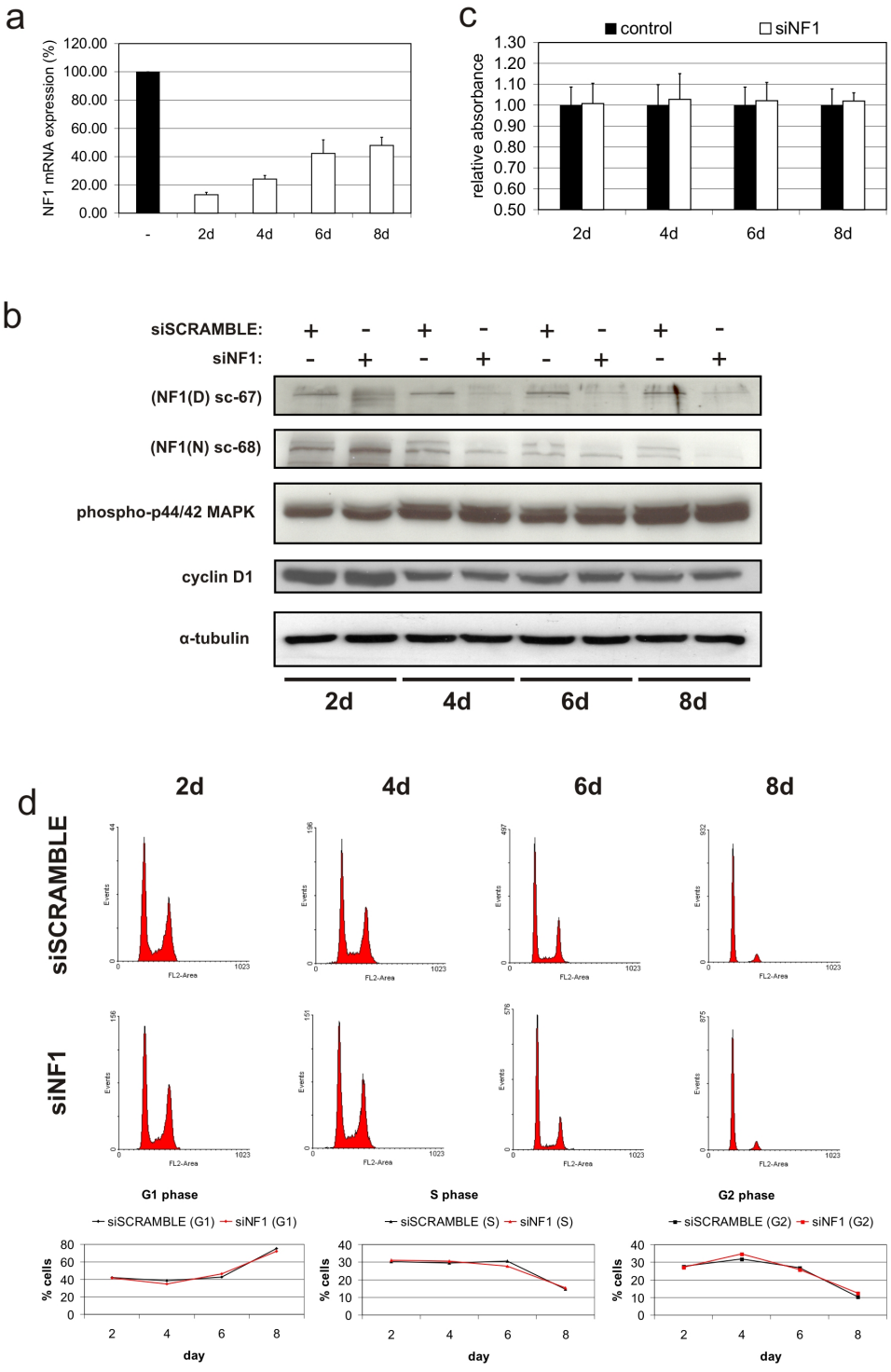
Figure legends

Figure 1. Effects of NF1 gene (product) suppression on cell proliferation and cell cycle distribution. (a) Quantitative real-time PCR analysis of *NF1* mRNA expression in control (siSCRAMBLE) siRNA (black bar) and siNF1 electroporated epidermal melanocytes (white bars; 2d, 4d, 6d and 8d). Data are expressed as mean +/- SEM of 3 replicates. (b) 6-10% PAGE immunoblot analysis of whole-cell lysates extracted from control (siSCRAMBLE) siRNA and siNF1 electroporated epidermal melanocytes after 2d (lane 1 and 2), 4d (lane 3 and 4), 6d (lane 5 and 6), 8d (lane 7 and 8). Neurofibromin was detected with an anti-neurofibromin C-terminal

antibody NF1(D) and N-terminal antibody NF1(N). Phospho-p44/42 MAPK (pERK) and cyclin D1 were detected with the appropriate antibodies. α-tubulin was used as loading control. (c) Effect of neurofibromin suppression on melanocyte proliferation after 2d, 4d, 6d and 8d was assessed by the MTS assay. Black bars represent control (siSCRAMBLE) siRNA electroporated melanocytes, white bars represent siNF1 electroporated melanocytes. Data are expressed as mean +/- sem of 2 replicates and are normalized to controls (d) Cell cycle analysis progression. Cells were harvested after 2d, 4d, 6d and 8d, fixed and stained for DNA content. The distribution (%) of cells in G1, S and G2 phase of the cell cycle are indicated.

Figure 1



Identification of amyloid precursor protein as novel neurofibromin interaction partner: expanding the role of neurofibromin in epidermal melanocytes and implications for NF1 etiopathology

Article V

Neurofibromatosis type 1 protein and amyloid precursor protein interact in normal human melanocytes and colocalize with melanosomes.

Sofie De Schepper [#], Joachim Boucneau [#], Wendy Westbroek, Mieke Mommaas, Ludwine Messiaen, Jean-Marie Naeyaert and Jo Lambert. *J Invest Dermatol* 126(3): p.653-659 (2006) ([#] first two authors equally contributed).

Copublished in the same issue (commentary article in *J Invest Dermatol* 126(3): p.547-550) and based on our study, Diwakar and Hornyak discussed the association between APP and neurofibromin, and their localization to melanosomes. They postulated and confirmed the potential importance of this association in the etiopathology of CALMs.

See related commentary on page 547

ORIGINAL ARTICLE

Neurofibromatosis Type 1 Protein and Amyloid Precursor Protein Interact in Normal Human Melanocytes and Colocalize with Melanosomes

Sofie De Schepper[1,5], Joachim M.A. Boucneau[1,5], Wendy Westbroek[2], Mieke Mommaas[3], Jos Onderwater[3], Ludwine Messiaen[4], Jean-Marie A.D. Naeyaert[1] and Jo L.W. Lambert[1]

The neurofibromatosis type 1 (NF1) gene product, neurofibromin, is known to interact with Ras, thereby negatively regulating its growth-promoting function. Although this is a well-established interaction, the discovery of other neurofibromin interacting partners could reveal new functional properties of this large protein. Using yeast two-hybrid analysis against a brain cDNA library, we identified a novel interaction between the amyloid precursor protein and the GTPase activating protein-related domain of neurofibromin. This interaction was further analyzed in human melanocytes and confirmed by immunoprecipitation and colocalization studies. In addition, we observed a colocalization of amyloid precursor protein and neurofibromin with melanosomes. Amyloid precursor protein has been proposed to function as a vesicle cargo receptor for the motor protein kinesin-1 in neurons. This colocalization of amyloid precursor protein and neurofibromin with melanosomes was lost in melanocytes obtained from normal skin of a NF1 patient. We suggest that a complex between amyloid precursor protein, neurofibromin, and melanosomes might be important in melanosome transport, which could shed a new light on the etiopathogenesis of pigment-cell-related manifestations in NF1.

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INTRODUCTION

Neurofibromatosis type 1 (NF1) is one of the most common autosomal dominant neurocutaneous disorders in man with a worldwide prevalence of roughly 1 in 3,500 individuals (Riccardi, 1981). The disorder primarily affects tissues of neural crest origin and exhibits a wide clinical expression spectrum, ranging from benign peripheral nervous system tumors (neurofibromas), optic pathway gliomas, skeletal dysplasia to pigmentary defects (café-au-lait spots, freckling, Lisch nodules). Apart from the fact that the disease is a heavy psychological burden, it is also accompanied by a number of complications such as an increased risk of developing malignancies and learning disabilities (De Schepper *et al.*, 2005). The disorder is caused by mutations of the *NF1* gene. This gene spans approximately 350 kb, is localized on chromosome 17q11.2, and has 60 exons. The transcript is 11–13 kb long and encodes a protein, called neurofibromin, of 2,818 amino acids (Viskochil *et al.*, 1993). Neurofibromin is a large protein expressed in numerous tissues with the highest expression levels being observed in neurons (Nordlund *et al.*, 1993). The only region of neurofibromin, which has been well characterized, is a central domain of about 360 amino acids showing a significant sequence and functional homology to the mammalian Ras-specific GTPase activating proteins (RasGAPs – eg $p120^{GAP}$). This domain is called the GAP-related domain (GRD) and increases the slow intrinsic GTPase activity of the growth regulator Ras up to 10^5-fold. A growing number of neurofibromin interacting or associating proteins and organelles have been described over the years. It has been shown that neurofibromin can bind to certain major cytoskeletal structures. Neurofibromin is able to associate with microtubules through its GRD and it has been proposed that this association is important for regulating the growth-promoting activity of Ras (Bollag *et al.*, 1993; Gregory *et al.*, 1993; Xu and Gutmann, 1997). An interaction switch between different cytoskeletal structures was described in telencephalic neurons, with neurofibromin exhibiting a biphasic differentiation-dependent expression pattern and a differential subcellular localization to the F-actin and microtubule cytoskeleton (Li *et al.*, 2001). In addition, a

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Abbreviations: APP, β-Amyloid precursor protein; GAP, GTPase activating protein; GRD, GAP-related domain; NF1, neurofibromatosis type 1

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stable association between the kinesin-1 heavy chain and neurofibromin has recently been shown (Hakimi *et al.*, 2002). Interaction with extracellular matrix-binding proteins such as the transmembrane heparan sulfate proteoglycan syndecan-2 has been suggested as a mechanism for localizing neurofibromin to specific domains of the plasma membrane, perhaps contributing to synaptic RasGAP activity or adhesion signaling in the brain (Hsueh *et al.*, 2001). Colocalization of neurofibromin with the mitochondria in cultured cell lines (Roudebush *et al.*, 1997) has been described, whereas localization to the smooth endoplasmic reticulum was seen in neurons (Nordlund *et al.*, 1993).

In order to find other functional interactions of neurofibromin, we set up a yeast two-hybrid screening of the neurofibromin-GRD and a brain cDNA library. In this report, we demonstrate that β-amyloid precursor protein (APP) interacts with the GRD of neurofibromin. We show that in human melanocytes, neurofibromin and APP colocalize with melanosomes. This interaction complex could be part of a melanosome transport/biogenesis regulating mechanism or a signaling complex or might be a mechanism for sequestering neurofibromin from the plasma membrane, where it functions as a negative regulator of Ras.

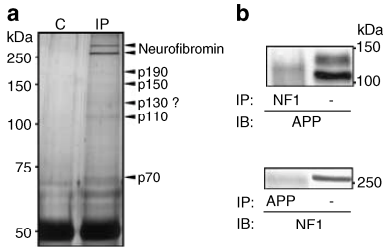

Figure 1. Identification of neurofibromin interacting proteins. (**a**) Neurofibromin in cell lysate of primary human epidermal melanocytes was immunoprecipitated with NF1 sc-67 antibody and the immunoprecipitate was analyzed by SDS-PAGE (6%) and silver stained. Lane C represents the rabbit polyclonal IgG isotype matched control, lane IP represents the neurofibromin immunoprecipitate. Molecular weight markers are shown on the left and on the right black arrows indicate the position of immunoprecipitated protein bands (p70, p110, p130, p150, p190). (**b**) Upper right figure: immunostaining with APP (CT15) antibody of NF1 sc-67 immunoprecipitate (left lane) compared to the total cell lysate (right lane). Lower right figure: immunoblotting with NF1 sc-67 antibody of APP (CT15) immunoprecipitate (left lane) compared to the total cell lysate (right lane). Molecular weight markers are shown in kDa.

RESULTS

Identification of APP as a neurofibromin-binding protein

Screening of possible direct interactions of neurofibromin with other proteins was performed using the GAL-4-based yeast two-hybrid vector system. The GRD of neurofibromin (bp 4,071–4,671) was screened against a commercially available brain cDNA library in the yeast strain *Saccharomyces cerevisiae* PJ-69-4A.

One of the interactions that remained after stringent selection with SD/-Leu-Trp-Ade, SD/-Leu-Trp-His, and the X-gal filter assay was identified as APP. Because of its important role in axonal transport in the brain (Kamal *et al.*, 2001) and its proposed role in melanosome transport and melanin release (Quast *et al.*, 2003), we decided to further investigate this interaction.

To further confirm and investigate the neurofibromin–APP interaction in human melanocytes, total melanocyte cell lysate was incubated with NF1(D) antibody to immunoprecipitate possible neurofibromin-containing protein complexes. As shown in Figure 1a, several bands with molecular masses between 70 and 200 kDa were obtained after silver staining of the gel (p70, p110, p130, p150, p190), but none of them were observed in the rabbit IgG isotype-matched control. The observed proteins p110 and p130 match the known molecular weights of immature (110 kDa) and mature (130 kDa) APP. Using the anti-APP antibody (CT15), APP was identified after Western blotting of a neurofibromin immunoprecipitate. The APP band, however, was located intermediate between the immature and mature form of APP. The reason for this remains unclear (Figure 1b, upper part). The reverse experiment was also performed to see whether neurofibromin co-immunoprecipitated with APP. This showed a 250 kDa band corresponding to the neurofibromin protein (Figure 1b, lower part).

Expression of *APP* and *NF1* gene (product) in cultured primary human epidermal melanocytes

We examined mRNA expression of APP and NF1 in normal human melanocytes using primers APP-F and APP-R (Quast *et al.*, 2003) and NF1-F and NF1-R. We saw the appropriate cDNA bands for the 751 and 770 isoforms of APP and for NF1 (94 bp) (Figure 2a). In addition, we checked the protein expression of APP using the polyclonal antiserum CT15 and of neurofibromin using the NF1(D) in normal human melanocytes by means of Western blot (Figure 2b). In human melanocyte protein extract a clear band representing neurofibromin was observed around 250 kDa. Two APP isoforms of around 110 and 130 kDa were detected, being the immature and mature isoforms of the protein, respectively.

Localization of APP and wild-type neurofibromin in cultured primary human epidermal melanocytes (healthy donor) and colocalization with melanosomes

As described earlier (Quast *et al.*, 2003) APP is mainly localized in a reticulum within the perinuclear region showing colocalization with the endoplasmic reticulum and premelanosomes and in granules at the tips of dendrites, representing mature melanosomes.

We investigated the distribution of APP and neurofibromin by indirect immunofluorescent labeling. In melanocytes, we observed the previously reported perinuclear staining pattern of APP associated with occasional staining of the dendrite tips (Figure 3b). Neurofibromin showed a granular staining pattern with perinuclear accentuation (Figure 3a).

Because both APP and neurofibromin displayed a similar organellar punctate staining pattern, a double labeling of neurofibromin and APP with the melanosomal marker NKI-beteb was performed.

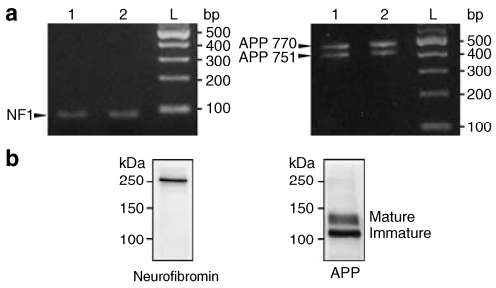

Figure 2. Expression of *NF1* gene (product) and *APP* gene (product) in primary human epidermal melanocytes. (**a**) Detection of NF1 (left) and APP (right) mRNA expression by RT-PCR analysis in primary human epidermal melanocytes (obtained from two different donors (1 and 2)), displaying the expression of both the 751 and 770 isoforms of APP. The same samples showed the appropriate 94 bp band (arrow) when analyzed with the exon 36–37 NF1 boundary primers. (**b**) Expression of neurofibromin and APP proteins was analyzed by SDS-PAGE (6%) followed by immunostaining with anti-neurofibromin (NF1 sc-67) and anti-APP (CT15) antibodies. Molecular weight markers are shown in kDa on the left. Note the immature and mature isoforms of APP.

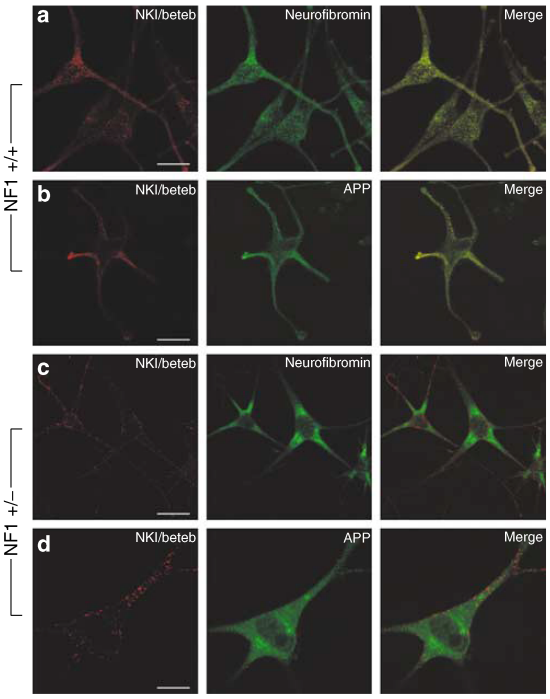

Figure 3. Subcellular distribution and localization of neurofibromin, APP and melanosomes in healthy donor ($NF1^{+/+}$) and NF1 patient ($NF1^{+/-}$) primary human epidermal melanocytes. Healthy donor melanocytes were cultured on coverslips for 24 hours, fixed and immunostained with (**a**) NKI-beteb and neurofibromin and (**b**) NKI-beteb and APP. The overlay panels show remarkable colocalization of neurofibromin especially in the perinuclear area and of APP in the perinuclear area and the dendrite tips. NF1 patient melanocytes were cultured on coverslips for 24 hours, fixed and immunostained with (**c**) NKI-beteb and neurofibromin and (**d**) NKI-beteb and APP. A clear absence of colocalization can be seen. Bars = 10 μm.

In melanocytes, NKI-beteb stains the melanocyte-specific glycoprotein Pmel17 or gp100 and displays a punctate staining pattern throughout the cell body and dendrites with accentuation around the nucleus and in the dendrite tips. APP shows a similar staining pattern especially around the perinuclear area, which colocalizes with premelanosomes, and in some of the dendrite tips, marking mature melanosomes. The staining was less intense in the cell periphery. Figure 3b shows high levels of colocalization between APP and NKI-beteb. Neurofibromin shows a perinuclear staining pattern with no obvious staining of the dendrite tips (Figure 3a). To study colocalization of neurofibromin and APP with melanosomes on an ultra-structural level, immuno-electron microscopy was performed. Figure 4a shows APP as 15 nm gold particles situated on organelles resembling melanosomes. Figure 4b shows neurofibromin as 15 nm gold particles situated on melanosomes. To make sure that the observed organelles are in fact melanosomes and not lysosomes or other organelles, a double immunostaining of neurofibromin (10 nm gold) and NKI-beteb (15 nm gold) was performed (Figure 4d). The same was performed for APP (Figure 4c). The results show remarkable colocalization on melanosomes. Owing to technical limitations, we were not able to perform double labelings of APP with neurofibromin.

An *NF1* gene mutation deranges the colocalization of both APP and neurofibromin with melanosomes

Double immunofluorescent staining was performed on cultured $NF1^{+/-}$ primary human epidermal melanocytes obtained from the skin of a severely affected NF1 patient with a deletion of two nucleotides of the *NF1* gene (3,525_3,526del2). Figure 3c shows almost complete loss of the overlap between neurofibromin and NKI-beteb, which implicates that mutant neurofibromin is unable to colocalize on melanosomes. Interestingly, Figure 3d indicates that this *NF1* gene deletion also affects the colocalization between APP and melanosomes.

Ultrastrastructural study with immuno-electron microscopy displayed less staining of neurofibromin (15 nm gold) (Figure 4f). The observed decrease in neurofibromin expression can be explained by the haploinsufficiency of the *NF1* gene locus due to NF1 heterozygosity. In the double immunostainings of APP (15 nm gold) and NKI-beteb (10 nm gold) (Figure 4 g), and neurofibromin (15 nm gold) and NKI-beteb (10 nm gold) (Figure 4 h), a specific and clear staining of melanosomes is visible. Neither APP nor neurofibromin localize to melanosome structures nor to any other identifiable structure.

DISCUSSION

Using yeast two-hybrid analysis against a brain cDNA library, we found a direct binding of APP with the GRD of neurofibromin. As our primary interest is in the pigment cell-related manifestations in NF1, we further investigated the APP–neurofibromin interaction in primary human epidermal melanocytes.

APP is a type I transmembrane cell surface protein with a large N-terminal extracellular part. Being known as the

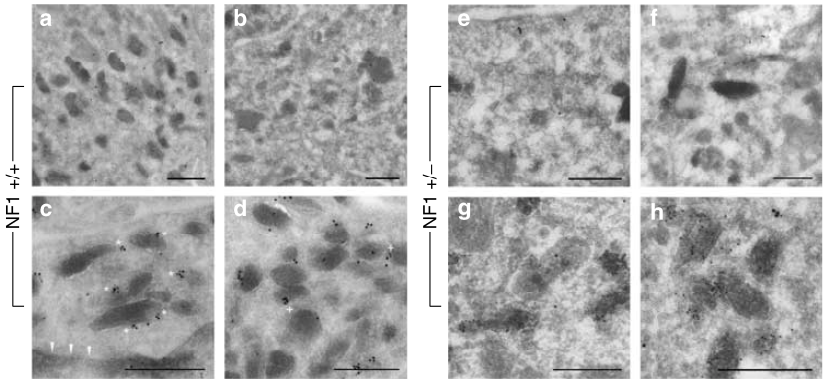

Figure 4. Ultrastructural distribution and localization of neurofibromin, APP and melanosomes in healthy donor (NF1 $^{+/+}$) and NF1 patient (NF1 $^{+/-}$) primary human epidermal melanocytes. Paraformaldehyde-fixed ultrathin cryosections of healthy donor primary human epidermal melanocytes were labeled with (**a**) *β*-amyloid antibody (15 nm gold) and (**b**) neurofibromin antibody (15 nm gold). Both APP and neurofibromin showed melanosomal localization. (**c**) A double labeling with NKI-beteb antibody (15 nm gold) and *β*-amyloid antibody (10 nm gold) revealed complexes associated on melanosomes (asterisk). The plasmamembrane of the dendrite is marked with arrows. (**d**) A double labeling with NKI-beteb antibody (15 nm gold) and anti-neurofibromin (10 nm gold) showed several melanosome-associated colocalization complexes (plus signs). Paraformaldehyde-fixed ultrathin cryosections of NF1 patient primary human epidermal melanocytes were labeled with (**e**) APP antibody (15 nm gold) and (**f**) neurofibromin antibody (15 nm gold). No melanosomal localization was seen. (**g**) The double labeling with NKI-beteb (10 nm gold) and APP (15 nm gold) and with (**h**) NKI-beteb (10 nm gold) and neurofibromin (15 nm gold) only showed clear labeling of the melanosomal marker NKI-beteb but no colocalization of (**g**) APP or (**h**) neurofibromin. Bars = 500 nm.

precursor of the amyloid-beta (A*β*) peptides involved in the pathogenesis of Alzheimer's disease, APP consists of different isoforms generated by alternative splicing of the *APP* gene. APP isoforms 751 and 770 (numbers referring to their length in amino acids) are found as the major translation products in, among others, the epidermis, whereas isoform 695 (lacking exon 7 and 8) is most abundant in the brain (Quast *et al.*, 2003). APP expression is especially high in epidermal melanocytes and therefore it has been suggested as an immunocytochemical marker for this cell type (Quast *et al.*, 2003). *β*-Amyloid, a neurotoxic peptide, is generated from APP through the action of *β*- and *γ*-secretases. However, the *α*-secretase pathway splits APP within the A*β* domain, producing a large amino-terminal non-amyloidogenic sAPP*α*, which functions as a regulator of dendrite motility and melanin release in epidermal melanocytes and melanoma cells (Quast *et al.*, 2003). Several interacting partners of APP have been recently described. The A*β* peptide binds to cyclin B1 and increases human cyclin-dependent kinase-1 activity (Milton, 2002). In addition, two-hybrid screening assays have detected three proteins that interact with the specific GYENPTY domain in the cytoplasmic tail of APP: Fe65, X11, and mDab1 (mammalian homologue of *Drosophila* disabled). Together with PAT1 (protein interacting with APP tail 1), they may provide a link between APP and the cytoskeleton (Herzog *et al.*, 2004). APP also functions as a matrix-binding protein interacting with perlecan, laminin, collagen type IV and endactin, and as a copper-binding protein important in neuronal copper homeostasis (Herzog *et al.*, 2004). Recent studies have demonstrated that the C-terminal part of APP forms a complex with neuronal kinesin-1 by direct binding to the tetratricopeptide repeat domain of the kinesin light chain (Kamal *et al.*, 2000) and that the JNK signaling scaffold protein JIP1b mediates this association (Inomata *et al.*, 2003). Interestingly, neurofibromin has also been shown to interact with kinesin-1, with cytoskeletal structures such as microtubules and with extracellular matrix-binding proteins such as syndecan-2 (Hsueh *et al.*, 2001; Li *et al.*, 2001). The direct association between neurofibromin and kinesin-1 was established by conventional and affinity chromatography, Western blot, and immunoprecipitation in the soluble and particulate fraction of HeLa extract and calf brain (Hakimi *et al.*, 2002). For the first time, we show a direct interaction between APP and the GRD of neurofibromin in the brain and in melanocytes, which is not surprising considering that both are linked to kinesin-1 and to the microtubular cytoskeleton and therefore must exhibit a close spatial relationship. In addition, we confirm the previously described colocalization of APP with melanosomes (Quast *et al.*, 2003) and show that neurofibromin is also present on the melanosomal membrane and forms a complex with APP. In neurons, APP is transported from the neuronal cell bodies towards the distal nerve terminals by kinesin-1-mediated axonal transport (Koo *et al.*, 1990; Sisodia *et al.*, 1993). As has already been suggested in a recent publication (Herzog *et al.*, 2004), we also believe that full-length APP aids the kinesin-mediated microtubular transport of melanosomes along the dendrites.

The existence of a complex between APP, neurofibromin, and melanosomes is especially interesting in light of the many pigment-cell-related manifestations seen in NF1, with café-au-lait macules being the major hallmark. Our experiments on NF1 $^{+/-}$ melanocytes obtained from the skin of a severely affected NF1 patient shows that the colocalization of both APP and neurofibromin with melanosomes is disturbed by the *NF1* gene mutation (Figures 3c, d and 4e–h).

NF1 melanocytes, carrying an *NF1* gene defect, contain many large melanosomal complexes and increased amounts

of melanin compared to normal human melanocytes (Martuza *et al.*, 1985; Kaufmann *et al.*, 1991). Melanin macroglobuli originate from the fusion of melanosome complexes with phagolysosomes. They are aggregates of melanosomes at various stages of melanization (De Schepper *et al.*, 2005). In this viewpoint, a mutated *NF1* gene product could, via the interaction with kinesin and APP, cause problems in melanosome biogenesis or transport or could even impair the interchange between melanosomes and lysosomes, leading to macromelanosome formation.

In addition, an interesting notion considering NF1 and Alzheimer's disease (both being common neurological disorders) is that neurofibromin and APP share their interaction with the molecular motor protein kinesin-1. In neuronal cells, processing of APP to β-amyloid can occur in an axonal membrane compartment, containing β-secretase and presenilin-1, transported by kinesin-1 (Kamal *et al.*, 2000, 2001). By demonstrating an interaction between neurofibromin and APP and localizing both constituents to the melanosomes of normal human melanocytes, we might expand the insight into vesicular trafficking. In light of the frequent cognitive defects in NF1 (mental retardation, learning disabilities, etc), it could be that mutation of the *NF1* gene product leads to an impaired kinesin-1-mediated protein and vesicle trafficking in neurons, perhaps distorting neurotransmitter transport or synaptic RasGAP activity in the brain (Hsueh *et al.*, 2001).

In summary, we provide new evidence for the existence of a complex consisting of melanosomes, neurofibromin, and APP, which might be relevant for the etiopathogenesis of several symptoms found in NF1, such as pigmentation or cognitive disorders.

MATERIALS AND METHODS

Cell culture

Primary human epidermal melanocyte cultures used for yeast two-hybrid screening, co-immunoprecipitation, indirect immunofluorescence and immuno-electron microscopy were obtained from neonatal foreskin of a healthy control individual or from an NF1 patient normal skin biopsy. Written informed consent was obtained from all patients and all described protocols/studies were approved by the medical ethical committee of the University of Ghent. The study was conducted in accordance with institutional guidelines on the Declaration of Helsinki Principles. Melanocyte cultures were established as described previously (Naeyaert *et al.*, 1991; Smit *et al.*, 1998). Briefly, the cells were cultured in Ham F10 (Gibco, Invitrogen Ltd, Paisley, UK) medium supplemented with 2.5% fetal calf serum, 1% Ultroser-G, 5 ng/ml basic fibroblast growth factor, 10 ng/ml endothelin-1, 0.33 nM cholera toxin, 5.3 nM D12-*O*-tetradecanoylphorbol-13-acetate and 0.033 mM 3-isobutyl-1-methylxanthine until subconfluency (80%).

Antibodies and reagents

Rabbit polyclonal antibodies NF1(D) (sc-67) (1/500) directed against the C-terminus of neurofibromin and NF1(N) (sc-68) (1/500) directed against the N-terminus were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal antibody NKI-beteb aimed against the (pre)melanosomal silver protein was obtained from Monosan (Uden, The Netherlands). Rabbit polyclonal antiserum CT15 directed against the C-terminus of APP (1/500) was kindly provided by Dr E. Koo (UCSD, San Diego, CA). Goat polyclonal amyloid antibody (sc-5399) (Santa Cruz Biotechnology, Santa Cruz, CA) was directed against the C-terminus and recommended for detection of β-amyloid and amyloid A4. Secondary rabbit anti-mouse and rabbit anti-goat immunoglobulins used for immuno-electronmicroscopy were obtained from Dako (DakoCytomation, Heverlee, Belgium). Horse radish peroxidase-conjugated anti-rabbit IgG antibody (1/4,000) and HRP-conjugated anti-mouse IgG antibody (1/3,000) were from Amersham Biosciences (Orsay, France).

RT-PCR

Using the RNeasy method (Qiagen, Leusden, The Netherlands) total RNA was extracted from normal human melanocytes. cDNA was prepared from total RNA using random primers (Invitrogen Ltd, Paisley, UK) and the Superscript II RT enzyme (Invitrogen Ltd, Paisley, UK). PCR amplification reaction was performed using normal human melanocyte cDNA, the Extaq enzyme (Takara Shuzo Co, Otsy, Shiga, Japan) and primers APP-F 5′-AAGCCACAGAGA GAACCACCAGCATT-3′ and APP-R 5′-GCTTGACGTTCTGCCTCTT CCCATT-3′ (Quast *et al.*, 2003) for APP and NF1-F 5′-ACGAGTGTC TCATGGGCAGAT-3′ and NF1-R 5′-ACTGTTGTAAGTGTCAGGTC CTTTTAAG-3′ for NF1. The thermal cycling conditions for APP were 30 cycles at 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 45 seconds, and conditions for NF1 were 35 cycles at 95°C for 30 seconds, 54°C for 30 seconds, and 72°C for 15 seconds. PCR products were size-separated on a 2% agarose gel and visualized by ethidium bromide staining.

Yeast two-hybrid analysis

Based on the full-length cDNA sequence of neurofibromin (GenBank accession number: NM_000267), the region of interest was PCR amplified using normal human melanocyte cDNA prepared from total RNA. This region is referred to as ''bait'' (major part of the GRD: bp 4,071–4,671). Primers used for amplification were baitF 5′-ATC AGTTCCTCCTCAGAATTC-3′ and baitR 5′-TACCTGATGCCTAGTCA TAAA-3′. The bait plasmid was constructed by directional insertion of PCR product in the cloning site (*Eco*RI/*Sma*I) of the pBD-Gal 4 vector (Stratagene, La Jolla, CA).

As an additional positive control syndecan-2 (Genbank accession number: XM_040582) was used (Hsueh *et al.*, 2001). This plasmid was constructed by *Bam*HI-*Eco*RI directional cloning into the pAD-Gal4-2.1 vector (Stratagene, La Jolla, CA) using the following primers: SynF 5′ GCGGAGTCGAGAGCAGAG-3′ and SynR 5′ TTAC GCATAAAACTCCTTAGTAG-3′. The inserts were sequence verified by Baseclear (Leiden, The Netherlands). The human brain cDNA library (''prey'') was inserted in the pAD-Gal4 vector. All constructs were tested for auto-activation. Internal positive and negative controls from Stratagene, being p53–pSV40 and pLamin–pSV40, respectively, were used. Briefly, the NF1-GRD-pBD-Gal4 construct and the commercial brain library construct (Clontech, BD Biosciences, Palo Alto, CA) were sequentially transformed in the PJ-69-4A yeast strain. Potential interactions were scored on selective SD/-Leu-Trp-Ade, SD/-Leu-Trp-His growth medium and the β-galactosidase filter assay. Only in cases where all three assays were positive, yeast DNA was prepared for further analysis. After transformation of the yeast DNA in supercompetent *E. coli*, the interacting proteins were sequence verified by Baseclear (Leiden, The Netherlands).

Immunoprecipitation

Normal human melanocytes were grown until subconfluency (80%) in D12-*O*-tetradecanoylphorbol-13-acetate supplemented Ham F10 medium (Gibco, Invitrogen Ltd, Paisley, UK). After washing the cells with dextrose supplemented phosphate-buffered saline, the cells were lysed for 30 minutes at 4°C with a mild lysis buffer (10 mM Tris, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 0.7% NP40, pH 7.4), containing protease inhibitors (10 μl/ml phenylmethylsulfonyl fluoride, 10 μl/ml Leupeptin, 10 μl/ml Aprotinin) and phosphatase inhibitors (20 μl/ml $NaVO_3$, 50 μl/ml $Na_4P_2O_7$, 10 μl/ml NaF 100 ×). Insoluble cell material was pelleted by centrifugation at 14,000 r.p.m. at 4°C for 10 minutes. The supernatant was collected and protein concentration was determined with the DC Protein Assay (BioRad, Hercules, CA). The supernatant was precleared with 25 μl protein A sepharose beads (Amersham Biosciences, Orsay, France) under shaking for 30 minutes at 4°C. After centrifugation, immunoprecipitation was performed by incubating precleared supernatant with either NF1(D) (sc-67) or APP antiserum CT15 under shaking for minimum 3 hours at 4°C, followed by the addition of 50 μl protein A sepharose beads and incubation for 1 hour at 4°C under shaking. Unbound proteins were removed by extensive washing. Immunoprecipitated proteins were extracted in 38 μl 1.5 × Laemmli Sample Buffer and boiled for 5 minutes. The immunoprecipitates were analyzed by Western blotting.

Western blotting

Immunoprecipitates or total cell lysate were solubilized and denatured by boiling in 1.5 × or 4 × Laemmli sample buffer respectively, containing 5% β-mercapto-ethanol and 0.25% bromophenol blue. Proteins were separated by SDS-PAGE (6% gel) and electro-blotted on to polyvinylidine diflouride membranes (Amersham Biosciences, Orsay, France) or silver stained using the Silver Stain Plus kit (Biorad, Hercules, CA). Membranes were blocked for 1 hour at room temperature in 5% non-fat dry milk in Tris buffered saline with 0.1% Tween (137 mM NaCl, 20 mM Tris, pH 7.6 with 0.1% Tween-20), followed by incubation with the appropriate primary antibody. Following several washing steps with Tris buffered saline with 0.1% Tween, membranes were incubated with the appropriate secondary antibody for 1 hour at room temperature. After final washing steps with Tris buffered saline with 0.1% Tween, bound antibodies were detected using the enhanced chemiluminescence detection system ECL + Plus (Amersham Biosciences, Orsay, France) according to the manufacturer's protocol.

Indirect immunofluorescence and confocal microscopy

Melanocytes were grown on coverslips and fixed for 20 minutes at room temperature with 3% paraformaldehyde in phosphate-buffered saline. After three washes in tris-buffered saline, cells were submerged for 10 minutes with 50 mM NH_4Cl in phosphate-buffered saline[E] (Eisen formulation) followed by three washes in tris-buffered saline. For permeabilization, cells were treated for 5 minutes with 0.2% Triton X-100 in phosphate-buffered saline. Double staining for neurofibromin and melanosomes was performed by first incubating for 2 hours at room temperature in a 1/40 NKI-beteb and a 1/50 NF1(D) dilution. After three washes in tris-buffered saline, the coverslips were incubated for 1 hour with an FITC-labeled rabbit anti-mouse (1/20) and a biotinylated donkey anti-rabbit (1/50) secondary antibody, respectively. Double staining for APP and melanosomes was performed by first incubating for 2 hours at room temperature in a 1/40 NKI-beteb and a 1/100 APP (CT15) dilution. After three washes in tris-buffered saline, the coverslips were incubated for 1 hour at room temperature in a biotinylated donkey anti-rabbit (1/50) and a FITC-labeled rabbit anti-mouse (1/20) antibody dilution. Slides were coverslipped in Prosan fluorescence mounting fluid and confocal image (1 μm) sections were obtained with a BioRad Confocal Laser Scanning Microscope (Radiance 2100 blue laser diode).

Immunogold transmission electron microscopy

Melanocytes were fixed for 24 hours at room temperature in 2% paraformaldehyde in 0.1 M PHEM buffer (60 mM Pipes/NaOH, 25 mM Hepes, 10 mM EGTA and 2 mM $MgSO_4$, pH 6.9–7.0) and processed for immunogold labeling as described elsewhere (Mommaas *et al.*, 1992a, b). Briefly, cells were pelleted and embedded in 12% gelatin, cut into 1 mmł cubes, cryoprotected in 2.3 M sucrose and snapfrozen in liquid nitrogen. For localization of neurofibromin and APP, ultrathin cryosections were incubated with the anti-neurofibromin antibody NF1(D) diluted 1/500 or anti-goat β-amyloid/amyloid A4 (1/500) antibody (with intermediate rabbit-anti goat step) or anti-APP (1/500) antibody (CT15), followed by 15 nm protein A-gold incubation (1/200). For colocalization with melanosomes a double labeling with the NKI-beteb antibody (dilution 1/300), after an intermediate incubation step with a secondary rabbit anti-mouse bridging antibody (1/200) and the NF1(D) (1/500) antibody was performed. For double labeling with NKI-beteb and anti-goat β-amyloid antibody, both an intermediate step with a secondary rabbit anti-mouse (1/200) and rabbit anti-goat (1/200) antibody, respectively, were necessary. All antibodies were incubated with 10 or 15 nm Protein A gold particles (1/200) for visualization. As negative controls the primary antibodies were omitted, solely including the secondary antibodies and 10 and 15 nm Protein A gold particles. After immunolabeling, sections were embedded and contrasted in methylcellulose/uranyl acetate and viewed with a Philips EM 410 electron microscope (Eindhoven, The Netherlands).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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General discussion and future perspectives

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Neurofibromatosis type 1 (NF1) is one of the most common neurocutaneous cancer predisposition syndromes known to humans and affects primarily neural crest-derived cells and tissues (Poyhonen et al., 1997). Fifty percent of NF1 patients have sporadic NF1 gene mutations, while the other half has a familial history for the disease (Friedman et al., 1999). The NF1 gene is approximately 350 kb of genomic DNA, generates a transcript of around 11-13 kb, codes for a protein (neurofibromin) of 2818 aa and is ubiquitously expressed (Li et al., 1995; Bernards et al., 1992; Marchuk et al., 1991).

NF1 patients present with typical hyperpigmentary cutaneous (café-au-lait macules [CALMs] and intertriginous ephelides) and hyperpigmentary non-cutaneous lesions (iris Lisch nodules) (see **chapter 1 – article I**), which could argue for a specific role of the NF1 gene (product) in regulating differentiation (melanin synthesis, melanosome biogenesis / transport, melanosome transfer) and / or proliferation of the pigment producing cell, the melanocyte. The vast majority of NF1 research has been mainly focused on the (peripheral nerve sheet) Schwann cell as central (neoplastic) cell involved in the etiopathogenesis of neurofibromas. These lesions pose a heavy physical and psychosocial burden, increasing the urge for an effective therapy. However, a closer look into the role of the (epidermal) melanocyte in other (more bearable) NF1 disease manifestations (e.g. CALMs) will only help in extending our knowledge of the function of this large tumor suppressor gene (product). The only major cellular function of neurofibromin that has been studied in detail and has been the primary link to its tumor suppressor properties, is the GTPase activating protein (GAP) function which can be ascribed to a centrally located region, called the GAP-related domain (GRD) (Xu et al., 1990).

[I] NF1 haploinsufficiency: when half is enough ...

An elegant approach – which has been applied in this study – to investigate the functional role of a gene and its corresponding protein in a particular (human) cell type, is to work with an in vitro cell system in which the gene of interest is mutated, and consequently produces an aberrant, reduced or absent protein. A potential mechanism that explains an abnormal phenotype arising as a result of the mutation of one allele (heterozygosity) is that of haploinsufficiency, i.e. the situation in which the total level of a gene product produced by a cell is about half of

the normal baseline levels and is not sufficient to permit the cell to function normally. The role of haploinsufficiency as a phenomenon of gene dosage reduction and sensitivity is often underestimated when it comes to its contribution to tumorigenesis (Santarosa and Ashworth, 2004; Wu et al., 2005). In many cases, heterozygous loss-of-function mutations can lead to the generation of tumors (in a haploinsufficiency context) that are frequently of later onset and less severe than the corresponding tumor carrying the homozygously mutated gene (e.g. p27, p53, Dmp1), while haploinsufficiency in other tumor suppressor genes is associated with early stages of the disease (e.g. hamartomatous polyp formation; PTEN, Smad4, Lkb1) (Quon and Berns, 2004; Cook and McCaw, 2000). These haploinsufficiency effects could be applied to e.g. neurofibroma formation in NF1 patients which starts to emerge around puberty and early adolescence (later onset) (see **chapter 1**). However, the question still remains as to whether the increased occurrence and presence of CALMs and hamartomatous iris Lisch nodules in NF1 (see **chapter 1**) can also be explained by NF1 haploinsufficiency, as these clinical manifestations are congenital or show an early onset. Some groups have suggested that embryological melanoblast migration defects (perhaps due to altered growth / survival / motogenic factor sensitivity) could lie at the basis of CALM etiopathogenesis in NF1 (reviewed in De Schepper et al., 2005).

When our present study was initiated, nothing was known on the effects of NF1 heterozygosity on total cellular gene / transcript expression. Congenital autosomal mutations of a tumor suppressor gene allele can induce profound cell and tissue-specific biological effects which are in part the result of altered gene expression and function. Loss-of-function mutations often provide clues to the role genes play in a cell or organism. How does NF1 heterozygosity (and consequently haploinsufficiency of the NF1 gene locus) affects the melanocyte and can NF1 heterozygosity tell us something more on the function of neurofibromin? To address this question, we used an in vitro NF1 heterozygous ($NF1^{+/-}$) primary human epidermal melanocyte system consisting of cells that were isolated and cultured from NF1 patient skin biopsies (lesional and non-lesional skin). This approach can be considered as an ultimate model system to learn more about the role of the NF1 gene in a cell-specific manner. We performed high-throughput cDNA microarray analysis to compare the effects of a mutated NF1 allele on the expression of the melanocytic transcriptome between NF1 wild type ($NF1^{+/+}$) melanocytes and NF1 heterozygous ($NF1^{+/-}$) melanocytes (see **chapter 2**). The particular experimental loop design used in this study enabled us to search for possible melanocyte-intrinsic lesional type-dependent effects (normally pigmented skin melanocytes versus hyperpigmented café-au-lait skin melanocytes). Microenvironmental effects due to heterotypic cellular interactions in the epidermal region and / or due to signaling through paracrine cytokine networks are not considered in this in vitro setup. Our data unraveled the fact that gene expression was mainly affected by genotypic differences (one normal – one mutated versus two normal NF1 alleles). The differential expression mainly involved genes regulating cell proliferation and cell adhesion. Several transcription factor genes, involved in melanocyte-lineage development, were downregulated in $NF1^{+/-}$ melanocytes. These were shown to be involved in the cis-regulated transcriptional regulation of dopachrome tautomerase (DCT) expression (see also **chapter 1**), a major melanocytic biomarker gene.

It has already been demonstrated in vitro that $p21^{Ras}$-GTP levels do not alter in a NF1 heterozygous melanocytic background (Griesser et al., 1995) and that these melanocytes strangely enough showed a reduced proliferative capacity (Kaufmann et al., 1991). In light of gene dosage sensitivity due to haploinsufficiency it was interesting to investigate if reduction in NF1 gene expression (e.g. by post-transcriptional downregulation) in normal melanocytes had a (similar) effect on melanocyte proliferation. We artificially suppressed NF1 gene (product) expression by siRNA-mediated RNA interference (see **chapter 3**). Primary human epidermal melanocytes were electroporated with specific siRNA molecules targeting the NF1 gene. Functioning as a true tumor suppressor protein, neurofibromin is able to decrease cellular proliferation by inhibiting its downstream effector $p21^{Ras}$.

However, in our study melanocytes did not show any proliferative changes upon reduction of endogenous neurofibromin, which was reflected by a lack of induction of phosphorylated ERK ($p21^{Ras}$ downstream effector) or cyclin D1 expression. The cell cycle profile also remained unchanged. These results suggest that neurofibromin is not the major regulator of melanocyte proliferation, at least in vitro.

Our work has primarily focused on the role of neurofibromin in an in vitro melanocytic cell system. We can only speculate on how a multicellular-multifactorial microenvironment influences the functional properties of neurofibromin in a single cell-type and how a cell-specific response propagates in its immediate surroundings. The contribution of genetic and / or epigenetic changes in non-tumor cells to tumorigenesis has only begun to be appreciated recently. Haploinsufficiency of the NF1 gene is required for tumor formation by NF1 homozygous mutant cells. A tractable mouse model of neurofibromas does exist and appears to be a usefull tool to study the role of Nf1 gene (product) in the context of heterotypic cell interactions in vivo. These conditional knock-out mice, which have a Nf1 heterozygous genotypic background essential for tumor formation, contain a floxed Nf1 allele that can be deleted by a Cre-recombinase transgene under the control of the Schwann cell-specific promotor, Krox-20 (Zhu et al., 2002). In this way the cell type (i.e Schwann cell) in which Nf1 gets deleted can be controlled, a situation that is not the case in chimeric mouse models that are partially composed of $Nf1^{-/-}$ cells (Cichowski et al., 1999). $Nf1^{+/-}$ mast cells were suggested as having a crucial role in progression of neurofibroma formation (ECM remodeling and angiogenesis) as it was shown that neurofibromin-deficient Schwann cells secreted elevated levels of stem cell factor (SCF) as migratory stimulus for mast cells within the tumor environment (Yang et al., 2003; Wu et al., 2005). In mice it has been shown that primary $Nf1^{+/-}$ astrocyte cultures failed to demonstrate a cell-autonomous growth advantage unless they were cocultured with C17 neuronal cells. This cell-context dependent increase in astrocyte proliferation was suggested to be sufficient for the development of astrocyte growth abnormalities in patients with NF1 (Gutmann et al., 1999). Whether there is a similar mechanism working in the epidermis that might explain the increased number of melanocytes in NF1 patients (see **chapter I – article 2**) needs to be further investigated. The observation that mono-cultures of $NF1^{+/-}$ melanocytes do not show this cell growth advantage (Kaufmann et al., 1991; own observations) supports this cell-context dependency. In vivo, epidermal melanocytes are indeed under the control of autocrine and paracrine cytokine networks and this cell context / cytokine dependent interaction between epidermal keratinocytes, dermal fibroblasts and epidermal melanocytes, is probably playing a major role in (hyper)pigmentary disorders (Imokawa et al., 2004). Hypersensitivity for growth factors (e.g SCF) due to Nf1 haploinsufficiency has already been demonstrated in mouse melanocytes and mast cells (Ingram et al., 2000). A relevant mouse model for CALMs (to study the role of neurofibromin in melanocytes in a cell context-dependent environment) does not exist and is probably difficult and perhaps impossible to generate. In order to study the functional role of neurofibromin in a more controlled physiological environment resembling the natural epidermis (mimicking heterotypic intercellular interactions), an initial 2-dimensional coculture system of epidermal melanocytes and keratinocytes could be used (Lei et al., 2002). We could introduce mixtures of NF1 heterozygous melanocytes and / or keratinocytes to study growth factor sensitivity (e.g. SCF) and its cellular effects in a haploinsufficient environment. This could teach us more on the exact role of neurofibromin in epidermal homeostasis. It has been shown that Nf1 haploinsufficiency increases mast cell proliferation and survival upon SCF treatment (Ingram et al., 2000). To further increase the epidermal resemblance, reconstructed skin could be an excellent laboratory working tool to address the question which genetic and epigenetic factors are potentially involved in normal epidermal homeostasis in general and more specific in the regulation of the epidermal melanocyte. CALMs are not considered as neoplastic or hyperplastic lesions as the basic tissue organisation of the epidermis remains intact compared to normal skin and does not present with the cellular heterogeneity of the complex tissue mass in a neurofibroma that once was a normal peripheral nerve. An attempt has already been made to construct a pigmented skin equivalent to study

hyperpigmentation in CALMs. However, this model for congenital hyperpigmentary disorders was insufficient due to the lack of terminal differentiation of keratinocytes (Okazaki et al., 2005). The recent progress in the field of epithelial culture techniques has allowed the development of culture systems in which reconstructed epidermis shows characteristics of morphological differentiation similar to those seen in vivo. This skin equivalent is the dead de-epidermized dermis (DDD) on which a mixture of keratinocytes, melanocytes, fibroblasts and / or mast cells are seeded (Rehder et al., 2004). Albeit, these skin equivalent models could be an excellent tool for the evaluation of the effects of several (physiological) proliferation and differentiation promoting agents on the function of neurofibromin, not only in the melanocyte but also in its interplay with the surrounding microenvironment.

[II] Protein-protein interactions: neurofibromin in the spotlight

Using yeast-two-hybrid analysis, co-immunoprecipitation studies and subcellular / ultrastructural confocal laser scanning and immuno-electron microscopy, we have identified and localized a novel protein-protein interaction between amyloid precursor protein (APP) and the GRD of neurofibromin (see **chapter 4**). Recent studies have been interpreted as consistent with the idea that APP serves as a kinesin-1 cargo receptor and plays a role in the anterograde movement of membranous (vesicular) cargos along microtubules (Kamal et al., 2000; Lazarov et al., 2005). We showed that both APP and neurofibromin were able to colocalize with melanosomes and demonstrated that this association with melanosomes was lost in $NF1^{+/-}$ melanocytes. This is an interesting observation in light of the hyperpigmentary defects seen in NF1 and suggests a specific role for neurofibromin in the regulation of melanosome transport / biogenesis in human epidermal melanocytes. The biological significance and interaction details of the neurofibromin–APP association need to be further clarified as the functional role of this interaction in specific cellular / tissue systems is currently unknown:

- Using site-directed mutagenesis (introducing substitution mutations) in combination with yeast-two-hybrid analysis and reporter gene assays, we can modify, screen and narrow down the GRD for key residues mediating the interaction with APP. The reverse can be done to find the direct binding sites in APP. Screening of a (large) cohort of NF1 patients with known missense mutations in their GRD can also help us determine the direct binding site mediating the interaction with APP.

- As we suggested that the interaction is potentially involved in the regulation of vesicle (e.g. melanosome) transport / biogenesis, this complex could have analogous functional properties in other vesicle transporting / secreting (neural crest-derived) cell types like neurons. A small percentage of NF1 patients present with mental redardation and other cognitive defects that could argue for an important role of this protein complex in neurotransmitter transport and release towards synaptic membranes. Interestingly, the motor protein kinesin-1 has been shown to be a link between NF1 – as a common neurological disorder – and Alzheimer's disease (Hakimi et al., 2002). Our finding that APP, which functions as a kinesin-1 cargo receptor, shows an interaction with neurofibromin not only strengthens this link but could provide basic biological insights into vital cellular processes (e.g. protein trafficking).

The large-scale identification of novel neurofibromin interacting or associating proteins will help extend our knowledge on the cellular function of this large tumor suppressor. State-of-the art proteomics techniques for the extensive analysis of subcellular localization and distribution of neurofibromin in subcellular structures could help us discover new biological insights and would open up many new ways for future investigations:

- By controlled permeabilisation and homogenization of melanocytes, followed by subcellular (organellar) fractionation using a combination of centrifugation approachtes (equilibrium density gradient), we can determine the amount of neurofibromin in specific organellar fractions (mitochondria, ER, nucleus, Golgi, melanosomes, etc.). In these purified organellar fractions we could search for organelle-typic neurofibromin interacting / associating proteins. This would advance our understanding of the role of neurofibromin. The development of isoform-specific antibodies against neurofibromin would greatly enhance the resolution of subcellular neurofibromin distribution and function.

- In combination with high-resolution proteomics (2-dimensional gel electrophoresis (2DGE) followed by mass spectrometry-based protein identification methods) we could map the intracellular and / or interorganellar localization and transport of different neurofibromin isoforms and their specifc interaction partners during key cellular events (e.g. melanosome biogenesis-transport-transfer, mitosis, apoptosis, etc.), under different physiological states, and in normal and diseased cells.

- Placing protein-protein interactions in a more physiological context, specifically with regard to (post)translational modification-mediated interactions (e.g. due to phosphorylation), could be done using the mammalian-two-hybrid system (MAPPIT; Mammalian Protein-Protein Interaction Trap; Eykerman et al., 2001), which allows to study protein interactions in mammalian cell types (in situ) in stead of a eukaryotic yeast background.

Clearly, the time has come for an organized effort to characterize subcellular proteomes in different types of human cells. In order to obtain a global comprehensive picture of the function of a gene product these new technologies will be invaluable tools. The use of proteomics to characterize proteins and their relation to subcellular organelles will provide new functional insights, and these data will become more complete and convincing when proteomic analyses will be combined with protein localization and protein knock-down studies. Perhaps neurofibromin will be in the spotlight soon ...

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Summary – Samenvatting – Résumé

Summary

Neurofibromatosis type 1 (NF1) is one of the most common genetic neurocutaneous disorders with a prevalence of approximately 1 in 3500 individuals. NF1 affects primarily neural crest-derived cells and tissues, and patients develop both benign and malignant tumors at an increased frequency. Affected individuals present with a variety of clinical signs of which cutaneous neurofibromas, café-au-lait macules and iris Lish nodules are cardinal hallmarks of the disease. Advances in the understanding of the molecular pathogenesis of clinical features in NF1 have resulted from the identification of the NF1 tumor suppressor gene and the elucidation of its function. The only extensively characterized functional domain is the centrally located GAP-related domain (GRD). Being a large protein, neurofibromin harbors several other (unidentified) functional domains, and dito cellular functions, which could extend our knowledge of how mutations of the NF1 gene cause specific NF1 disease manifestations. An interesting observation in NF1 is the presence of specific hyperpigmentary cutaneous (café-au-lait macules, intertriguinous ephelides) and hyperpigmentary non-cutaneous lesions (iris Lisch nodules), which could argue for a specific role of neurofibromin in regulating cellular differentiation and / or proliferation of the pigment producing cell, the melanocyte.

The major goal of this work was to gain more insight into the functional role of the NF1 gene and its corresponding gene product neurofibromin in primary human epidermal melanocytes.

Initially we investigated the role of the NF1 gene by assessing the effect of NF1 gene heterozygosity on the expression of the melanocytic transcriptome. Using cDNA microarray analysis we compared human NF1 heterozygous ($NF1^{+/-}$) epidermal melanocytes to NF1 wild type ($NF1^{+/+}$) epidermal melanocytes, both being isolated and cultured from normally pigmented skin and hyperpigmented lesional café-au-lait skin. We found that gene expression was affected most strongly by genotype and less so by lesional type, probably due to in vitro conditions. A high number of transcription factor genes, among which a specific subset important in melanocyte lineage development, were downregulated in a cis-regulatory network governing the activation of the melanocyte-specific dopachrome tautomerase (DCT) gene. Although the results presented have been obtained with a restricted number of patients (one NF1 patient and one control) and using cDNA microarrays that may limit their interpretation, the data nevertheless addresses for the first time the global effect of a heterozygous NF1 gene on the expression of the human melanocyte transcriptome and has generated several interesting candidate genes helpful in elucidating the etiopathology of café-au-lait macules in NF1 patients.

In light of gene dosage sensitivity due to haploinsufficiency it was of course interesting to see if reduction in NF1 gene expression in normal melanocytes had an effect on melanocyte proliferation. We artificially suppressed NF1 gene (product) expression by siRNA-mediated RNA interference. Primary human epidermal melanocytes were electroporated with specific siRNA molecules targeting the NF1 gene. Functioning as a true tumor suppressor protein, neurofibromin is able to decrease cellular proliferation by inhibiting its downstream effector $p21^{Ras}$. However, in our study melanocytes did not show any changes in cell proliferation upon reduction of endogenous neurofibromin, which was reflected by a lack of induction of phosphorylated ERK ($p21^{Ras}$ downstream effector) or cyclin D1 expression. The cell cycle profile also remained unchanged. These results suggest that neurofibromin does not play a major role in regulation of melanocyte proliferation, at least in vitro.

The discovery of novel neurofibromin interacting proteins can reveal new functional properties of this large tumor suppressor protein. We identified and characterized amyloid precursor protein (APP) as a new neurofibromin-associating protein in primary human epidermal melanocytes. Using yeast two hybrid analysis against a brain cDNA library, we identified a novel interaction between APP and the GRD of neurofibromin. This interaction was further analyzed in human melanocytes and confirmed by immunoprecipitation and colocalization studies. In addition, we observed colocalization of APP and neurofibromin with melanosomes, the melanin-containing organelles of the melanocyte. APP has been proposed to function as a vesicle cargo receptor for the motor protein kinesin-1 in neurons. This colocalization of APP and neurofibromin with melanosomes was lost in melanocytes obtained from a severly affected NF1 patient. We suggest that a tripartite complex between APP, neurofibromin, and melanosomes might be important in melanosome biogenesis, transport and transfer.

In conclusion, this study has demonstrated the use of several molecular approaches (large scale – small scale) to investigate and extend the role of the NF1 gene and its corresponding protein neurofibromin in primary human epidermal melanocytes. Gaining better insight in the functional role of a (defective) gene (product) adds to a better understanding of the etiology of NF1 and to the progress in treatment of these non-life threatening hyperpigmentary anomalies.

Samenvatting

Neurofibromatose type 1 (NF1) behoort tot één van de meest voorkomende genetische, neurocutane ziekten met een prevalentie van 1 op 3500 individuen. Voornamelijk cellen en weefsels afkomstig van de neurale lijst zijn aangetast en patiënten ontwikkelen met een verhoogde frequentie zowel goed- als kwaadaardige tumoren. Getroffen personen vertonen een verscheidenheid aan klinische tekens waarvan cutane neurofibromen, café-au-lait vlekken en Lisch nodules de voornaamste kenmerken zijn van de ziekte. Dankzij de ontdekking van het NF1 tumor suppressor gen en de opheldering van zijn functie is vooruitgang geboekt in onze kennis van de moleculaire pathogenese van deze klinische kenmerken in NF1. Het enige functionele domein dat in detail is gekarakteriseerd, is het centraal gelegen GAP-related domain (GRD). Als groot eiwit heeft neurofibromine nog verschillende andere (niet geïdentificeerde) functionele domeinen, en dito cellulaire functies, die onze kennis kunnen uitbreiden van hoe mutaties in het NF1 genproduct aanleiding geven tot specifieke NF1 ziektebeelden. Een interessante waarneming in NF1 is de aanwezigheid van specifieke gehyperpigmenteerde cutane (café-au-lait vlekken, sproeten in oksels en lies) en gehyperpigmenteerde niet-cutane letsels (Lisch nodules in de iris), die een argument zouden kunnen zijn voor de rol van neurofibromine als regulator van cellulaire differentiatie en / of proliferatie van de pigment producerende cel, de melanocyt.

Met deze studie wilden we meer inzicht verwerven in de functionele rol van het NF1 gen en zijn corresponderend genproduct neurofibromine in primaire humane epidermale melanocyten.

Initiëel hebben we de rol van het NF1 gen onderzocht door de effecten na te gaan van NF1 heterozygositeit op de expressie van het melanocytair transcriptoom. Door middel van cDNA microarray analyse vergeleken we gekweekte humane NF1 heterozygote melanocyten ($NF1^{+/-}$) met NF1 wild type ($NF1^{+/+}$) melanocyten, die zowel uit normaal gepigmenteerde als gehyperpigmenteerde huid van een lesionele café-au-lait vlek geïsoleerd en gekweekt waren. We zagen dat een genexpressie het meest beïnvloed werd door het genotype, niet zozeer door het lesionele huidtype waaruit de melanocyten gekweekt waren. Een grote hoeveelheid transcriptiefactoren vertoonden een verlaagde expressie, waaronder een specifieke subset die belangrijk is in de ontwikkeling van de melanocyt, en waren betrokken in een cis-regulerend netwerk dat instond voor de activatie van het melanocyte-specifieke gen dopachrome tautomerase (DCT). Hoewel de resultaten afkomstig zijn van een beperkt aantal patiënten (één NF1 patiënt en 1 gezonde controle) in combinatie met cDNA microarray analyse die als limiterend

zou kunnen aanzien worden, worden hier toch voor de eerste keer de effecten van NF1 heterozygositeit nagegaan op de expressie van het melanocytair transcriptoom. Hierbij zijn tevens een interessante set van kandidaatgenen naar voor gekomen die zouden kunnen meehelpen in de opheldering van de etiopathogenese van café-au-lait vlekken in NF1 patiënten.

In het licht van gendosage gevoeligheid die te wijten is aan haploinsufficientie, was het natuurlijk interessant om na te gaan of reductie in NF1 genexpressie in normale melanocyten een effect had op proliferatie van melanocyten. Kunstmatig onderdrukten we de expressie van het NF1 gen(product) door middel van siRNA-gemedieerde RNA interferentie. Primaire humane epidermale melanocyten werden geëlectroporeerd met NF1 gen-specifieke siRNA moleculen. Als een echt tumor suppressor eiwit is neurofibromine in staat celproliferatie te verminderen door zijn downstream effector $p21^{Ras}$ te inhiberen. In onze studie daarentegen zagen we geen enkele verandering in de celproliferatie na reductie van endogeen neurofibromine, en dat werd weerspiegeld door een gebrek aan inductie van ERK phosphorylatie of cycline D1 expressie. Het profiel van de celcyclus bleef ook onveranderd. Deze resultaten suggereren dat neurofibromin geen grote rol speelt in de regulatie van melanocyt proliferatie, tenminste in vitro.

De ontdekking van nieuwe neurofibromine-interagerende eiwitten kan nieuwe functionele eigenschappen van dit groot tumor suppressor eiwit ontsluieren. Wij hebben het amyloid precursor proteïne (APP) geïdentificeerd en gekarakteriseerd als een nieuw neurofibromine interagerend eiwit in primaire humane epidermale melanocyten. Via ‘yeast two hybrid’ analyse tegen een ‘brain’ cDNA bibliotheek, identificeerden we een nieuwe interactie tussen APP en de GRD van neurofibromine. Deze interactie werd verder geanalyseerd in humane melanocyten en bevestigd met co-immunoprecipitatie en colokalisatie studies. Daarenboven observeerden we een colokalisatie tussen APP en neurofibromine met melanosomen. APP wordt in neuronen gezien als vesikel cargo receptor voor het motoreiwit kinesin-1. Dergelijke colokalisatie tussen APP en neurofibromine met melanosomen was verloren in melanocyten afkomstig van de normale huid van een NF1 patiënt. Wij suggereren dat een tripartiet complex tussen APP, neurofibromine en melanosomen van belang kan zijn in melanosome biogenese, transport en transfer.

Tot slot kunnen we stellen dat we in deze studie verschillende moleculaire benaderingen (large scale – small scale) hebben toegepast om de rol van het NF1 gen en zijn overeenkomstig eiwit neurofibromine in primaire humane epidermale melanocyten te onderzoeken en uit te breiden. Een beter inzicht verschaffen in de functionele role van een (defect) gen(product) kan ons alleen maar helpen in een betere kennis van de etiologie van NF1, en kan bijdragen aan de vooruitgang in de behandeling van deze niet-levensbedreigende gehyperpigmenteerde lesies.

Résumé

La Neurofibromatose de type 1 (NF1) est une des maladies génétiques neurocutanées les plus communes touchant 1 individu sur 3500. La maladie NF1 affecte de manière prédominante les cellules et les tissus dérivés de la crête neuronale, et les patients développent des tumeurs bénignes et malignes à une fréquence augmentée. Les individus affectés présentent une variété de signes cliniques dont les neurifbromes, les taches café-au-lait et les nodules de Lisch caractérisant la maladie. L'avance des connaissances de la pathogénie moléculaire des caractéristiques cliniques de NF1 résultent de l'identification du gène suppresseur de tumeur NF1 et sa fonction. Le seul domaine fonctionnel bien caractérisé est le domaine nommé GRD (GAP related domain), situé au centre du gène. Etant une protéine de grande taille, la neurofibromine contient plusieurs autres domaines fonctionnels non-identifiés, ainsi que des fonctions cellulaires, qui pourraient nous amener à mieux comprendre comment les mutations dans le gène NF1 peuvent causer les manifestations spécifique à la maladie. Une observation intéressante dans NF1 est la présence des lésions hyperpigmentées cutanées (les taches café-au-lait, les taches de rousseur axillaires / inguinales) et les lésions hyperpigmentées non-cutanées (les nodules de Lisch). Celles-ci suggèrent un rôle spécifique de la neurofibromine dans le contrôle de différentiation et de prolifération de la cellule contenant la mélanine, le mélanocyte.

Le but majeur de cette étude était mieux comprendre le rôle fonctionnel du gène NF1 et de son produit, la neurofibromine, dans les melanocytes primaires issus d'épiderme humain.

Nous avons commené par examiner le rôle du gène NF1 en évaluant l'effet de hétérozygosité du gène NF1 sur l'expression du transcriptome mélanocytique. Utilisant l'analyse des microarrays d'ADNc, les melanocytes primaires issus d'épiderme humain présentant une hétérozygosité du gène NF1 ($NF1^{+/-}$) ont été comparés à des melanocytes primaires issus d'épiderme humain contenant un gène NF1 sauvage ($NF1^{+/+}$). Les deux types de mélanocytes ont été isolés et cultivés hors de peau pigmentée normale et de peau hyperpigmentée lesionelle (des taches café-au-lait). Nous avons trouvé que l' expression du gène était fortement déterminée par le génotype et moins par le type de lesion, probablement liés aux conditions in vitro. Un grand nombre de gènes de facteur de transcription, parmi lesquels un (sous-)ensemble spécifique important au développement du lignage melanocytaire, ont été réprimés dans un réseau cis-régulatoir gouvernant l'activation du dopachrome tautomerase (DCT) qui est un gène mélanocytaire spécifique. Bien que les résultats présentés ont été obtenus dans un

nombre limité de patients (un patient NF1 et un patient contrôle) et en utilisant des microarrays d'ADNc, pouvant limiter l'interprétation des données, celles-ci ont adressé pour la première fois l'effet global de hétérozygosité du gène NF1 sur l'expression du transcriptome mélanocytaire humain et ont démontré les produits de plusieurs gènes candidats intéressants pouvant aider à expliquer l'étiopathologie des taches café-au-lait dans les patients NF1.

Tenant compte de la sensibilité de dosage du gène en raison de la haplo-insufficiance, il était intéressant de voir si une réduction de l'expression du gène NF1 dans des melanocytes normales avait un effet sur la prolifération des melanocytes. Pour cela nous avons éliminé artificiellement l'expression du (produit de) gène NF1 par l'ARN interférence medié par les siRNAs. Des melanocytes primaires issus d'épiderme humain ont été electroporées avec des molécules de siRNA spécifiques ciblant le gène NF1. Fonctionnant comme une protéine suppresseur de tumeur, la neurofibromine pouvait diminuer la prolifération cellulaire en inhibant son effecteur $p21^{Ras}$ dans plusieurs lignes cellulaires (dérivantes des patients NF1). Cependant, les melanocytes n'ont pas montrés de changement dans leur prolifération cellulaire après réduction de la neurofibromine endogène et ceci se réflétait par un manque d'induction de phosphorylation de la protéine ERK (effecteur de $p21^{Ras}$) et de la protéine cyclin D1. Le profil du cycle cellulaire restait également inchangé. Ces résultats suggèrent que la neurofibromine ne joue pas de rôle majeur dans le règlement de prolifération des melanocyte, tout du moins in vitro.

La découverte des nouvelles intéractions protéine-protéine avec la neurofibromine pourraient révéler de nouvelles propriétés fonctionnelles de cette protéine suppresseur de tumeur. Nous avons identifié et charactérizé une nouvelle intéraction protéine-protéine entre la protéine précurseur de la protéine beta-amyloid (APP) et la neurofibromine dans les melanocytes primaires issus d'épiderme humain. En utilisant le système de deux hybrides en levure dans une bibliothèque d'ADNc du cerveau humain, nous avons identifié une nouvelle intéraction protéine-protéine entre l'APP et la GRD de la neurofibromine. De plus, cette intéraction a été analysée dans les mélanocytes humains et confirmée (par rapport à des) dans des études de coprécipitation immunologique et colocalisation. Nous avons égalament observé une colocalisation entre l'APP, la neurofibromine et les mélanosomes, qui sont les organelles mélanocytaires contenant de la mélanine. L'APP a été proposée de fonctionner comme un récepteur de cargaison vésiculaire pour la protéine moteur kinésine-1 dans les neurones. Cette colocalisation entre l'APP, la neurofibromine et les melanosomes était absente dans les melanocytes obtenus d'un patient NF1 gravement atteint. Nous suggérons qu'un complexe tripartite entre l'APP, la neurofibromine, et les melanosomes est important dans la biogenèse, le transport et le transfert mélanosomique.

En conclusion, cette étude a démontré l'usage de plusieures approches moléculaires (large scale – small scale) pour examiner et étendre le rôle du gène NF1 et son produit du gène, la neurofibromine, dans les melanocytes primaires issus d'épiderme humain. Gagnant mieux la perspicacité dans le rôle fonctionnel d'un (produit du) gène (défectueux) ajoute à une meilleure compréhension de l'étiologie de NF1 et au progrès dans le traitement de cettes anomalies hyperpigmentées.

Curriculum Vitae

Personalia

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Education

1991 – 1997	Secondary school training (Latin-Sciences), Sint-Leocollege, Brugge, Belgium
1997 – 1999	Bachelor degree in Biology, Ghent University, Ghent, Belgium
1999 – 2001	Masters degree in Biotechnology, Ghent University, Ghent, Belgium Thesis: Development of a novel method to detect SNPs using DNA chips Promotor: Prof. Dr. Marc Zabeau, Department of Plant Genetics, Ghent University, Ghent, Belgium
2001 – 2002	Graduate in Complementary Studies of Informatics, Ghent University, Ghent, Belgium Thesis: Development of the website Formal Methods Europe Promotor: Prof. Dr. Ir. Rik Van De Walle, Department of Electronics and Information Systems, Ghent University, Ghent, Belgium
2003	Certificate Course Statistical Inference
2002 – 2006	PhD training in the laboratories of the Dermatology Research Unit (DRU), Department of Dermatology, Ghent University, Ghent, Belgium

Publications

[A] Articles in journals with peer-review system and international distribution

- De Schepper, S.[#], **Boucneau, J.[#]**, Lambert, J., Messiaen, L., and Naeyaert, J. M. (2005). Pigment cell-related manifestations in neurofibromatosis type 1: an overview. *Pigment Cell Res* 18(1): p.13-24. ([#] first two authors equally contributed) (IF=3.000)

- **Boucneau, J.**, De Schepper, S., Vuylsteke, M., Van Hummelen, P., Naeyaert, J. M., and Lambert, J. (2005). Gene expression profiling of cultures human NF1 heterozygous ($NF1^{+/-}$) melanocytes reveals downregulation of a transcriptional cis-regulatory network mediating activation of the melanocyte-specific dopachrome tautomerase (DCT) gene. *Pigment Cell Res* 18(4): p.285-299. (IF=3.000)

- De Schepper, S.[#], **Boucneau, J.**[#], Westbroek, W., Mommaas, M., Messiaen, L., Naeyaert, J.M., and Lambert, J. (2006). Neurofibromatosis Type 1 Protein and Amyloid Precursor Protein interact in Normal Human Melanocytes and colocalize with Melanosomes. *J Invest Dermatol* 126(3): p.653-659 ([#] first two authors equally contributed) (IF=4.238)

- De Schepper, S., **Boucneau, J.**, Vander Haeghen, Y., Messiaen, L., Naeyaert, J.M., and Lambert, J. (2006). Café-au-lait spots in Neurofibromatosis type 1 and in healthy control individuals: hyperpigmentation of a different kind? *Arch Dermatol Res* 297(10): p.439-449 (IF=1.217)

- **Boucneau, J**., De Schepper, S., Van Geel, M., Naeyaert, J.M., and Lambert, J. RNAi-mediated suppression of endogenous neurofibromin does not modulate ERK1/2 phosphorylation or the cell cycle in primary human epidermal melanocytes. (article in preparation)

[B] Abstracts of congress reports

- New interactions of the NF1 gene product, neurofibromin, in human brain and in melanocytes. De Schepper, S., **Boucneau, J.**, Westbroek, W., Lambert, J., Naeyaert, J.M. 11th European Society for Pigment Cell Research (ESPCR), Ghent, Belgium, 2003.

- Real-time quantitative PCR detection of NF1 and pigmentation-related gene expression in café-au-lait spots and normal skin of NF1 patients. De Schepper, S., Lambert, J., **Boucneau, J.**, Messiaen, L., Naeyaert, J.M. 10th European Neurofibromatosis Meeting, Turku, Finland, 2003.

- Neurofibromatosis type 1 (NF1) gene (product) expression in normal and café-au-lait skin of healthy controls and NF1 patients. De Schepper, S., **Boucneau, J.**, Naeyaert, J.M., Lambert, J. 12th European Society for Pigment Cell Research (ESPCR), Paris, France, 2004.

- Is there a potential role for dermal mast cells in the hyperpigmentary mechanism of café-au-lait maculae in Neurofibromatosis type 1 patients? **Boucneau, J.**, De Schepper, S., Naeyaert, J.M., Lambert, J. 34th European Society for Dermatological Research (ESDR), Vienna, Austria, 2004.

- Gene expression profiling of cultured human NF1 heterozygous (NF1$^{+/-}$) melanocytes reveals downregulation of a transcriptional cis-regulatory network mediating activation of the melanocyte-specific dopachrome tautomerase (DCT) gene. **Boucneau, J.**, De Schepper, S., Vuylsteke, M., Van Hummelen, P., Naeyaert, J.M., Lambert, J. 11th European Neurofibromatosis Meeting, Göteborg, Sweden, 2005.

- Second hit mutation analysis in melanocytes, keratinocytes and fibroblasts obtained from NF1 café-au-lait spots. De Schepper, S., Messiaen, L., Claes, K., Maertens, O, **Boucneau, J.**, Naeyaert, J.M., Lambert, J. 19th International Pigment Cell Conference (IPCC), Reston, Virginia, USA, 2005.

- RNAi-induced suppression does not modulate the cell cycle in cultured primary human epidermal melanocytes. **Boucneau, J.**, De Schepper, S., Van Gele, M., Naeyaert, J.M., Lambert, J. (will be presented by Lambert, J.). 67th Annual Meeting of the Society for Investigative Dermatology (SID), Philadelphia, Pennsylvania, USA, 2006.

Oral presentations

- NF1 and melanocytes. 1st NF1 workshop: wetenschappelijke onderzoeksgemeenschap FWO Vlaanderen, Molecular and Cellular mechanisms in NF1, Leuven, Belgium, January 10th – 11th, 2005.

- Neurofibromin interacts with amyloid precursor protein in normal human epidermal melanocytes and colocalizes with melanosomes: implications for NF1 etiopathology. 2nd NF1 workshop: wetenschappelijke onderzoeksgemeenschap FWO Vlaanderen, Molecular and Cellular mechanisms in NF1, Leuven, Belgium, December 21st – 22nd, 2005.

Participation to meetings, congresses and workshops

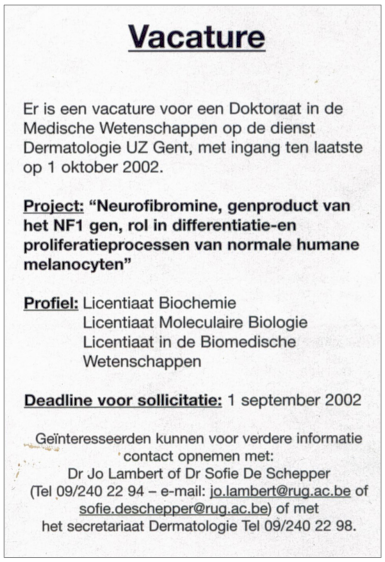
- Analis Confocal Microscopy Workshop, June 5th, 2003 (Ghent, Belgium)
- Special Federation of Biochemical Societies (FEBS) meeting on Signal Transduction, July 3rd – 8th, 2003 (Brussels, Belgium)
- 3rd VIB MicroArray Users Group meeting, September 10th, 2003 (Leuven, Belgium)
- 11th European Society for Pigment Cell Research (ESPCR) meeting, September 17th – 20th, 2003 (Ghent, Belgium)
- Wetenschapsdag vakgroep Inwendige Geneeskunde, Universitair Ziekenhuis Gent, January 22nd, 2004 (Ghent, Belgium)
- Life – a 'Nobel' story – symposium, April 28th, 2004 (Brussels, Belgium)
- 34th European Society for Dermatological Research (ESDR) meeting, September 9th – 11th, 2004 (Vienna, Austria)
- 4th VIB MicroArray Users Group meeting, November 18th – 19th, 2004 (Jette, Belgium)
- 11th European Neurofibromatosis meeting – Families and Professionals together, July 7th – 10th, 2005 (Gothenborg, Sweden)
- 1st NF1 workshop: wetenschappelijke onderzoeksgemeenschap FWO Vlaanderen, Molecular and Cellular mechanisms in NF1, January 10th – 11th, 2005 (Leuven, Belgium)
- Wetenschapsdag vakgroep Inwendige Geneeskunde, Universitair Ziekenhuis Gent, January 20th, 2005 (Ghent, Belgium)
- 2nd NF1 workshop: wetenschappelijke onderzoeksgemeenschap FWO Vlaanderen, Molecular and Cellular mechanisms in NF1, December 21st – 22nd, 2005 (Leuven, Belgium)

Grants – prizes – awards

- ASBL Neurofibromatose: "Project concerning learning difficulties in NF1 patients" – September 2nd, 2003 (financial credits: 1,000 euro)
- ESDR Travel Grant 2004 (financial credits: 500 euro)
- Reiskrediet Fonds voor Wetenschappelijk Onderzoek – Vlaanderen (FWO): "11th European Neurofibromatosis meeting – July 7th – 10th, 2005 (Gothenborg, Sweden)

Dankwoord

Gent, 4 juli 2002. Na de proclamatie aan de faculteit Toegepaste Wetenschappen – ik had een extra jaar aanvullende studies in de informatica gevolgd – fietste ik langs de Karel Ledeganckstraat waar ik een jaar eerder afgestudeerd was als licentiaat in de Biotechnologie. Ik wist niet wat de toekomst mij zou brengen. Het onderzoeksvuur brandde blijkbaar nog steeds, weliswaar op een laag pitje. Toen, heel toevallig, bladerde ik enkele dagen later in het magazine 'Gent Universiteit' (editie mei – juni 2002) en las ik op pagina 22 de vacature die de volgende 4 jaar van mijn leven zou bepalen.



Vacature

Er is een vacature voor een Doktoraat in de Medische Wetenschappen op de dienst Dermatologie UZ Gent, met ingang ten laatste op 1 oktober 2002.

Project: "Neurofibromine, genproduct van het NF1 gen, rol in differentiatie-en proliferatieprocessen van normale humane melanocyten"

Profiel: Licentiaat Biochemie
Licentiaat Moleculaire Biologie
Licentiaat in de Biomedische Wetenschappen

Deadline voor sollicitatie: 1 september 2002

Geïnteresseerden kunnen voor verdere informatie contact opnemen met:
Dr Jo Lambert of Dr Sofie De Schepper
(Tel 09/240 22 94 – e-mail: jo.lambert@rug.ac.be of sofie.deschepper@rug.ac.be) of met het secretariaat Dermatologie Tel 09/240 22 98.

Op 1 september 2002 vertrok de doctoraatstrein!

We zijn vandaag 1 maart 2006, de trein is bijna 4 jaar aan het bollen en het eindstation is in zicht.

Niettegenstaande de vermoeidheid zwaar de kop opsteekt (het neerschrijven en bundelen van 4 jaar werk gaat niet over één nacht ijs), wil ik hier toch de tijd nemen om iedereen te bedanken die ervoor heeft gezorgd dat de trein voortdurend op het juiste spoor bleef rijden en die heeft meegeholpen om op de gepaste tijdstippen de wissels te verzetten om de koers bij te sturen richting eindstation.

Eerst en vooral wil ik mijn promotoren bedanken, prof. dr. Jean-Marie Naeyaert en prof. dr. Jo Lambert. Professor Naeyaert, ik ben u heel dankbaar dat ik de kans heb gekregen om in uw vakgroep wetenschappelijk onderzoek te mogen doen. De interdisciplinaire kruisbestuiving in de wetenschappen die u hoog in het vaandel draagt, en uw heldere kijk, gedrevenheid en kritische ingesteldheid heb ik enorm geäpprecieerd en werkten enorm motiverend. Jo, een grote dank gaat uit naar jou. In alle drukte ben je steeds bereid geweest tijd vrij te maken om de resultaten en de vorderingen van het onderzoek te bespreken. Ik ben vereerd dat ik heb mogen bijdragen aan de uitbouw van jullie onderzoeksgroep. Continue steun en vertrouwen in mijn onderzoek. Dank voor het secuur nalezen van dit werk en de nuttige tips en correcties. Bedankt voor alles, ik vond het een eer!

Sofie (collega NF1 specialist), de eerste keer dat ik je heb ontmoet was tijdens de selectieronde voor kandidaten voor bovenstaande vacature. NF1 onderzoek was onze band. Ik heb de aangename samenwerking, je professionaliteit en collegialiteit enorm geäpprecieerd. Ik wens je heel veel succes met je doctoraat en de uitbouw van je verdere carrière. Het ga je goed!

En waar zou ik gestaan hebben zonder de hulp van 2 uitmuntende laboranten van onze onderzoeksgroep. Bedankt Martine ('*Melanocyten te koop, volledige uitverkoop, wie moet er hebben!*'), dankzij jou heeft de celcultuur alvast geen (of althans veel minder) geheimen meer voor mij. Jij zorgde er steeds voor dat allerhande huidcellen optimaal konden doorgroeien en dat wij nooit zonder 'grondstoffen' zaten voor experimenten. Marie-Chantal, jij hield de labo's draaiende en coördineerde de verhuis van onze met nieuwe naam voorziene onderzoeksgroep (DRU - Dermatology Research Unit) naar de Medical Research Building. Jij kon overal inspringen als er hulp nodig was. Bedankt voor alles. Ik wil ook Mireille, onze eerste post-doc, bedanken. Jij kwam pas later in onze research unit maar door je spontaniteit en gedrevenheid leek het alsof je er al van bij het begin bij was. Het was aangenaam om samen te werken en vele interessante discussies te voeren. Nog veel

succes met je project. *'Dien UV lampe, ojje dadde aanzet, da bloast nogal é!'* Karolientje, ook jij zwoegde doorheen je doctoraat. Op het moment dat ik dit schrijf zit je in de States. Veel (wetenschappelijk) plezier en succes in Washington. Ook jij bent er bijna! En natuurlijk wil ik ook mijn andere collega's doctoraatsstudenten bedanken, Barbara en Hilde, het was aangenaam samenwerken en veel succes in de toekomst.

Ik bedank ook alle mensen van de dienst huidziekten (polikliniek 6) voor de aangename samenwerking en de hulp die ik van jullie heb gekregen voor grotere en kleinere probleempjes: Liliane, An, Anthoine, Rita, Sofie, Francine, Katia, Nanja ... Ook ons twee-koppige IT-team wil ik bedanken. Merci Peter voor alle hulp als het systeem het eens liet afweten. Bedankt Yves voor de software-technische inbreng in bepaalde artikels en de interessante discussies. De kunst van het parapente-vliegen, de beste windrichtingen en opstijghellingen zijn nu geen mysterie meer.

Ik wil hier ook de collega's van het labo Experimentele Cancerologie bedanken. Tot vóór de verhuis van onze onderzoeksgroep was ons labo daar ondergebracht. Bedankt Ancy, Olivier, Barbara, Lara, Veerle, Georges, Rik, Arlette voor de leuke tijd daar! Eveneens wil ik Anouk en Liesbeth bedanken voor hun vrijgemaakte tijd en hulp bij de confocale microscoop. Ann, Sarah en Peggy van de vakgroep Reumatologie, bedankt voor de introductie in de FACS-wereld en alle hulp daarbij.
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En 'last but not least' wil ik mijn ouders en broer bedanken. Ma, Pa, 'Pierre', jullie hebben mij altijd de vrijheid gegeven te kiezen en te doen wat ik wilde. Jullie waren mijn toeverlaat en steun in iets moelijkere tijden en jullie hebben me altijd aangemoedigd er 100% voor te gaan. Hoewel ik het misschien weinig liet blijken, ben ik jullie oneindig dankbaar.

Bedankt iedereen,

Joachim Gent, Juni 2006

2006

Faculty of Medicine & Health Sciences

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Dermatology Research Unit